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THE
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PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

The executive committee of the Board of Trustees of the Marine Biological Laboratory has authorized the publication of the following resolution. It was read by Dr. E. G. Butler at the annual meeting of the Corporation on 16 August, 1968, and heartily approved by that meeting.

DONALD P. COSTELLO

Managing Editor of THE BIOLOGICAL BULLETIN

1951–1968

On the occasion of his retirement as Managing Editor of THE BIOLOGICAL BULLETIN the members of the Corporation of the Marine Biological Laboratory wish to express to

DONALD P. COSTELLO

their deep sense of gratitude for his devoted service to this journal. Beginning with Volume 100, he has been in charge of the editorial affairs of THE BIOLOGICAL BULLETIN for thirty-six volumes, covering a period of eighteen years. In the position of Managing Editor he has shown a fine sense of discrimination in the selection of manuscripts for publication; never being content with the average, always desirous of obtaining only those of highest quality. While giving attention to the maintenance of the traditional scope of the journal, he has continually been alert to changing emphasis, to new developments and the employment of new research techniques within the various research fields. For his watchfulness in all things pertaining to THE BIOLOGICAL BULLETIN, for his staunch devotion to highest standards and for the wisdom he has displayed at all times, the members of the Corporation of the Marine Biological Laboratory are grateful.

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MAJOR ENVIRONMENTAL FACTORS INDUCING THE TERMINATION OF LARVAL DIAPAUSE IN *CHAOBORUS AMERICANUS* JOHANNSEN (DIPTERA: CULICIDAE)[1]

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Diapause occurs in a great variety of arthropods and characteristically involves a cessation of differentiation. The developmental arrest may either encompass the whole animal as in egg, larval, and pupal diapause, or reside primarily in the germ cells, as in adult reproductive diapause. Arrest is usually accompanied by a metabolically quiescent state, and often involves the production of protective enclosures such as special egg cases, larval hibernacula, or pupal cocoons. However, in temperate climates, some immature aquatic insects overwinter in a state of diapause but appear as active as non-diapausing summer animals. Examples include *Metriocnemus* (Paris and Jenner, 1959), *Chironomus* (Engelmann and Shappirio, 1965), and *Chaoborus,* the subject of the current paper.

Chaoborids are noted for their tracheal air bladders (probably hydrostatic organs, Damant, 1924) and their voracious appetite. Lacking any thoracic appendages, chaoborids capture prey with prehensile antennae and swallow them with minimal mastication. *C. americanus* larvae in the vicinity of Ann Arbor, Michigan, swim beneath the winter ice of shallow ponds and kettle holes. A major question thus arises concerning the environmental factors involved in the maintenance and termination of a developmental arrest in this otherwise active animal.

Materials and Methods

Animals in the terminal larval instar were obtained November 11, 1965 and January 22, 1966 from a small stagnant woodland pond in Ann Arbor, Michigan. They were placed in Precision Scientific model 805 incubators at 5 ± 1° C equipped with 40 Watt cool white fluorescent lamps regulated by standard Lumenite timers. Animals from each day's collection were maintained *en masse* on short day (light:dark = 8:16) until used for experimentation. All experiments were run in pond water strained through at least three layers of cotton cloth at 25 ± $1\frac{1}{2}$° C with 10 or 20 animals in a 3 oz jar, 25 in a 7 oz jar, or individually in $\frac{1}{2}$ oz flint glass creamers (Table I). Long (light:dark = 17:7) or short (light:dark = 8:16) daylength was provided. Fed experimental animals received an excess supply of *Culex pipiens* larvae.

The resumption of development may first be discerned by the appearance of a

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[1] Aided by PHS Grant GM-06101.

[2] Appreciation is extended to David G. Shappirio for his constant consideration and advice concerning the research here presented.

TABLE I

Summary of food and photoperiod experiments

	n	Ct	Exp	No/Con	Size Con	%P
Long day, fed	51	1/23/66	1/24/66	20,20,10	4 oz	94
	51	1/23/66	2/7/66	25	7 oz	98
	50	1/23/66	2/18/66	1	½ oz	98
	48	1/23/66	9/4/66	1	½ oz	92
Long day, starved	50	11/20/65	1/22/66**	20,20,10	4 oz	20
	50	1/23/66	2/7/66***	25	7 oz	50
	49	1/23/66	2/18/66	1	½ oz	39
	54	1/23/66	8/7/66	1	½ oz	4
	39	1/23/66	8/31/66	1	½ oz	11
	46	1/23/66	9/4/66	1	½ oz	4
Short day, starved	50	11/20/65	1/22/66	20,20,20	4 oz	0
	50	1/23/66	1/23/66	25	7 oz	0
	51	1/23/66	4/4/66	1	½ oz	6
	42	1/23/66	8/7/66*	1	½ oz	5
	40	1/23/66	11/1/66	1	½ oz	5
Short day, fed	50	1/23/66	1/31/66	20,20,10	4 oz	2
	50	1/23/66	2/7/66	25	7 oz	4
	48	1/23/66	4/20/66	1	½ oz	44
	47	1/23/66	8/7/66*	1	½ oz	47
	47	1/23/66	11/1/66	1	½ oz	49

All experiments run at 25°C using *Culex pipiens* for food. Short day, 8:16; long day, 17:7; n, sample size; Ct, date caught; Exp, date placed on experimental conditions; No/Con, number of animals in each container; Size Con, size of containers used in fluid ounces; %P, per cent pupating. All experiments were run until all animals either pupated or died except *, **, and *** which were terminated after 26, 34, and 38 days, respectively.

third pair of air sacs on the anterioventral portion of the animal. These sacs become the exterior air sacs of the pupa and appear about eight hours prior to the pupal moult. Pupation itself was used as an indicator of development since: (1) the internal sacs were not always readily identifiable *en masse* or in large numbers of individual containers, (2) there is a striking transformation at pupation due to change in external form and change from a horizontal to vertical orientation, (3) very few larvae in which the pupal air sacs appeared failed to pupate, and (4) ecdysis and orientation change at pupation are almost instantaneous.

EXPERIMENTAL RESULTS

(1) *Long-term maintenance of stocks*

Animals maintained in the short day, low temperature, starved stocks would eat if fed; but, no larvae, fed or starved, ever developed under these conditions. Animals appeared to remain healthy from November 1965 until September–October 1966 when they began to die in large numbers. Some animals, however, were still alive through December 1966.

(2) *Effect of warming*

Sample populations of 40–51 animals were removed from the stocks and placed under short day conditions without food at 25° C. In all cases, no more than 6% development was observed (Fig. 1b).

(3) *Effect of food and long day*

Sample populations of 48–51 animals were removed from the stocks, placed under long day conditions at 25° C, and fed. 92–98% of these animals developed in each case (Fig. 1a).

(4) *Effect of food alone or long day alone*

When sample populations of 39–54 animals were removed from the stocks and placed on long day but without food, a wide spread of 4–50% development resulted. This distribution fell into two distinct groups: one set of 20–50% pupating popula-

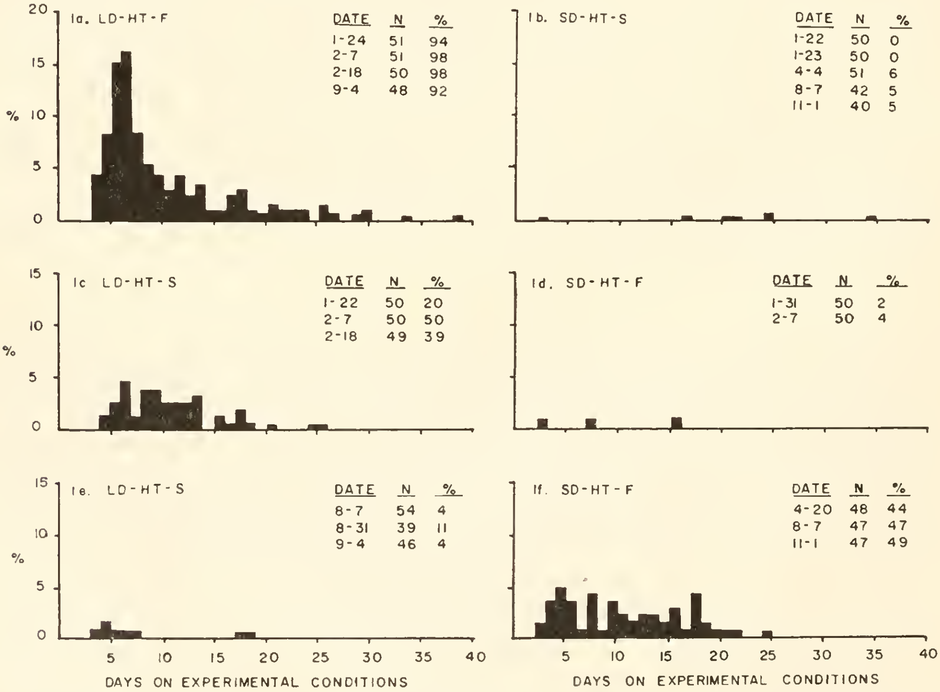


FIGURE 1. Pupation of *Chaoborus* under different combinations of light and photoperiod. Ordinate: average per cent of animals pupating that day; abscissa: number of days on experimental conditions; LD, long day; SD, short day; HT, 25° C; F, fed; S, starved; Date, date in 1966 that the experiment was initiated; N, sample size of replicate; %, total per cent pupation of that replicate.

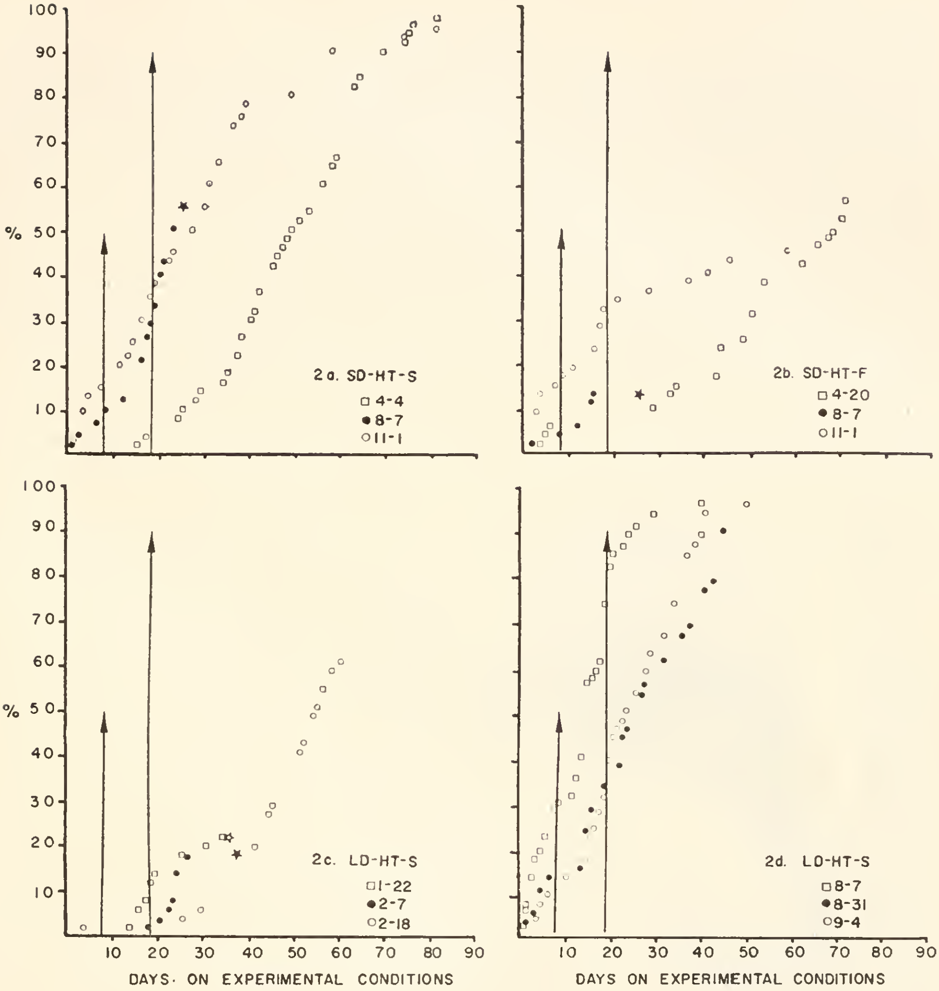


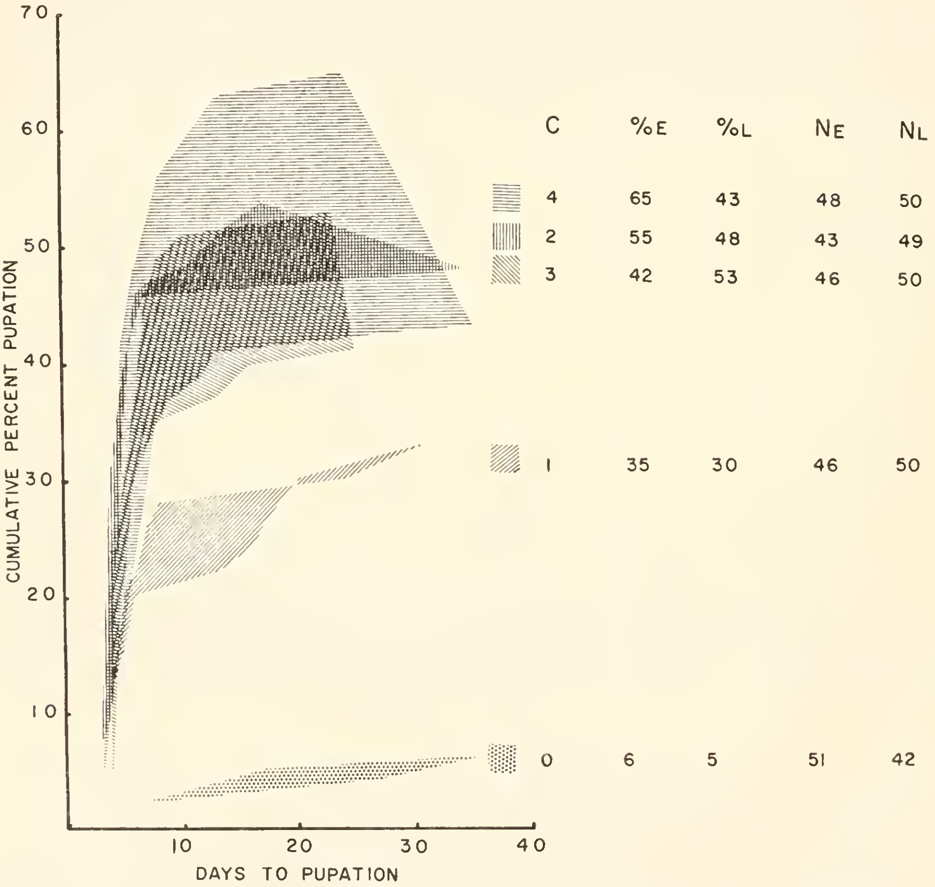
FIGURE 2. Death distribution of *Chaoborus* under different light and food combinations. Vertical arrows indicate average 50% (shorter) and 90% (taller) development under long day fed conditions. A star indicates that the experiment was not carried to completion but was terminated on that day. Ordinate, cumulative per cent dying; abscissa, number of days on experimental conditions. Other abbreviations as in Figure 1.

tions in January and February 1966 (Fig. 1c), the other set of 4–11% pupating populations in August and September 1966 (Fig. 1e).

Likewise, sample populations of 47–50 animals fed but kept on short day also showed a wide spread of 2–49% development with distributions falling into two distinct groups. In this case, however, low percentage pupating populations were observed in January and February (Fig. 1d) and intermediate percentage pupating populations in April, August, and November (Fig. 1f).

(5) *Induction time*

The rapid response of animals on long day with food prompted the question of how many long day cycles with food were necessary to induce development. Consequently, two experiments were run, one in April and one in August. In each case, animals were removed from the stocks and placed on long day with food at 25° C for 1, 2, 3, or 4 days. The food was then removed and animals were placed on short day at 25° C and observed until all animals either pupated or died. Fed and starved controls on short day were run as well. One long day cycle with food was found to induce development in 30–35% of the animals. Two or more cycles induced 42–65% development but a wider scatter was observed (Fig. 3). The continuously fed controls on short day showed 44 and 48% development but reached this level very gradually after prolonged feeding for two weeks.



	C	%E	%L	NE	NL
▤	4	65	43	48	50
▥	2	55	48	43	49
▧	3	42	53	46	50
▨	1	35	30	46	50
▒	0	6	5	51	42

FIGURE 3. Induction time, showing ranges of duplicate experiments. C, number of cycles long day fed before short day starved; %E and %L, total per cent pupation in April and August experiments, respectively; Ne and Nl, sample size of April and August experiments, respectively.

Discussion

C. americanus overwinters in a state of diapause rather than a thermally maintained quiescence. This conclusion follows from the observed lack of a developmental response at 25° C under three conditions: first, short day without food throughout the year (Fig. 1b); second, short day with food in the winter (Fig. 1d); and third, long day without food after prolonged chilling in the laboratory (Fig. 1e). There remains the possibility that animals in all but the long day fed experiments may not have developed due to differential mortality. An examination of the recorded death distributions (Fig. 2), however, indicates that only in one long day starved experiment in August (Fig. 2d) could the absence of development be explained by a high death rate.

Developmental dependence upon long day for a hibernating animal is neither new nor surprising. The interesting aspects of response to the environment in *C. americanus* are the unusually rapid response and the interrelationship between long day and food. A comparison of long day without food with short day fed experiments in winter (Fig. 1c–d) and in spring and summer (Fig. 1e–f) shows that long day is required for any significant development in the winter but not in the spring or summer; conversely, food is required for any significant development only in the spring and summer but not in the winter. There thus appears to be a shift in the major cue relied upon for the resumption of development.

The physiological basis for this observed shift may be the result of adaptation to a temperate environment. Shallow ponds such as those in which *C. americanus* characteristically occur (Cook, 1956) undergo repeated thawing and refreezing in the spring, accompanied by rapid plankton blooms and gradual predator buildups. For an aerial insect overwintering in such an environment, an extremely important consideration is the chance refreezing of the pond which could produce drowning at adult emergence or act as a barrier to oviposition. Hence, it is proposed that they depend upon the most reliable geophysical phenomenon, light, until a time of the year, determined by selection, during which refreezing becomes a lesser danger than consumption by predators or a lack of food. While refreezing may select for delayed termination of diapause, two factors would select for rapid development. First, a prolonged larval stage would be increasingly subject to predation, especially by Hemiptera and Odonata. Second, a prolonged larval stage may decrease the number of summer generations, thereby decreasing the potential of the individual to mix its genotype.

Although light may be the major stimulus in the winter and food in the spring or summer, it should be emphasized that the effect of both food and long day together is not additive but synergistic (Fig. 1). This type of response in most insects may be subject to speculation by anyone familiar with insect endocrine systems; but, *Chaoborus* has a distinctive neurosecretory system. In the larval brain, Füller (1960) found three sets of neurosecretory cells, of which the middle set gave off axons to the ventral abdominal nerve cord in which there were definite neurosecretory tracts, and the posterior set gave off axons to the corpus cardiacum and corpus allatum. Abdominal stimulation at room temperature of animals previously maintained at 5° C produced a general discharge of neurosecretory material from both sets of neurosecretory cells and the corpus cardiacum. The greatest amount of secretion was observed in larvae in which the pupal air sacs were most highly developed, *i.e.*, immediately prior to pupation. Thus one may suggest that while light is influencing the brain directly in a manner illustrated by Williams (1963), food may be acting via the ventral abdominal ganglia immediately ventral to the gut, with the two stimuli being integrated by the brain. Indeed, preliminary experiments now indicate that food is acting via a neuro-endocrine reflex independent of nutrition.

Chaoborus differs significantly from other "active" diapausing insects such as *Chironomus* and *Metriocnemus* in that the latter feed upon detritus but *Chaoborus* is primarily a carnivore. *Chironomus* maintained at 5° C and short day in the laboratory, for example, terminate diapause in early winter only when exposed to warm temperature and long day (Engelmann and Shappirio, 1965) ; in late winter without long day but with warm temperature (Shappirio, personal communication) ; and in mid-summer spontaneously on short day at 5° C (Bradshaw, unpublished observations). Food, however, was present at all times and was contributing an unknown inductive effect. *Chaoborus* has thus provided a unique opportunity to study the food component in "active" diapausing insects.

SUMMARY

1. The termination of larval diapause in *C. americanus* is cued by simultaneous long day and food.

2. One long day cycle with food elicits development in a significant proportion of the population.

3. A shift in the major developmental stimulus from long day during the winter to food after prolonged chilling in the laboratory was observed and is hypothesized to be an adaptation to life in shallow ponds in temperate climatic regions.

4. The action of food may occur via the ventral abdominal nervous system as described by Füller (1960) with ultimate neuro-endocrine control of development residing in the brain.

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THE TUBIFICIDAE (ANNELIDA, OLIGOCHAETA) OF CAPE COD BAY WITH A TAXONOMIC REVISION OF THE GENERA *PHALLODRILUS* PIERANTONI, 1902, *LIMNODRILOIDES* PIERANTONI, 1903 AND *SPIRIDION* KNOLLNER, 1935[1]

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The marine Tubificidae are an abundant, widely distributed, but poorly known group of Oligochaeta. The most recent account dealing with the fauna of the Cape Cod area is that of Moore (1905) who recognized seven species, mainly from littoral and estuarine environments. Material from a survey of the fauna of Cape Cod Bay which is being conducted by the Biotic Census of the Systematics-Ecology Program was found to contain a number of species which Moore did not see, five of these being new to science.

Two of the species described here, *Phallodrilus obscurus* nov. sp., and *Peloscolex intermedius* nov. sp., are very closely related to some species described by earlier workers and differ from them only in minor details. However, two considerations are thought to be sufficient justification for describing them as new species. Firstly, the available descriptions of closely related species are generally poor and cannot be augmented at present as type material is not available. Secondly, even when descriptions are adequate and type series are available for comparison, material tends to be from widely separated geographical areas, and without more extensive collections it is impossible to decide whether the differences observed are due to a wide intraspecific variation over the species range, discontinuous variation meriting subspecific rank, or real specific differences. In this situation, where the taxa are morphospecies in the sense of Cain (1954), it is proposed that the least confusing action from the nomenclatural point of view, where doubt exists, is to keep specific limits narrow, erect new species names and synonomize, if necessary, when type material becomes available for the old species.

At the generic level some rearrangments have been necessary to clarify definitions and to attain consistency between them. The major characters on which this has been based are the nature and position of the prostate glands and the form of the atria. Thus in *Clitellio* Savigny, 1820 (Fig. 1a) the prostate gland is lacking, or is a diffuse layer covering the long cylindrical atrium, while in *Limnodriloides* Pierantoni, 1903 (Fig. 1b) the prostate is a discrete organ with the attachment to the relatively short atrium localized to a small area. In *Spiridion* Knollner, 1935 (Fig. 1c) the attachment is further localized so that the prostate is truly pendunculate and joins the atrium apically rather than subapically to medially as in *Limnodriloides*. *Aktedrilus* Knollner, 1935 is synonomized with *Phallodrilus* Pierantoni, 1902 (Fig. 1d) on the basis of their common possession of two thickly-stalked prostate glands, attached to each short cylindrical atrium.

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[1] Contribution No. 162 from the Systematics-Ecology Program.

METHODS

The Biotic Census of Cape Cod Bay is a continuing long term investigation of the fauna of this area. Samples are taken uniformly over the area of the Bay at predetermined locations on a grid of nautical mile squares. Quantitative samples are being taken with a Smith-McIntyre grab in the middle and at each corner of every alternate quadrat. Material is being washed through a series of screens down to 0.5 mm mesh diameter, narcotized in propylene phenoxytol, fixed in formalin and stored in 80% ethyl alcohol. Oligochaeta from 27 completed samples (1.0 mm screen fraction) and two unsorted samples (0.5 mm screen fraction) were examined.

The anatomy of the Tubificidae was studied microscopically by one, or a combination of, three methods: a) worms were lightly stained in acetic haematoxylin and mounted whole in Canada Balsam, b) the genitalia were dissected out of stained worms using sharpened needles and forceps, and the parts mounted in Canada Balsam, c) the genital regions of the worms were sectioned at 7 μ and stained in haematoxylin and eosin.

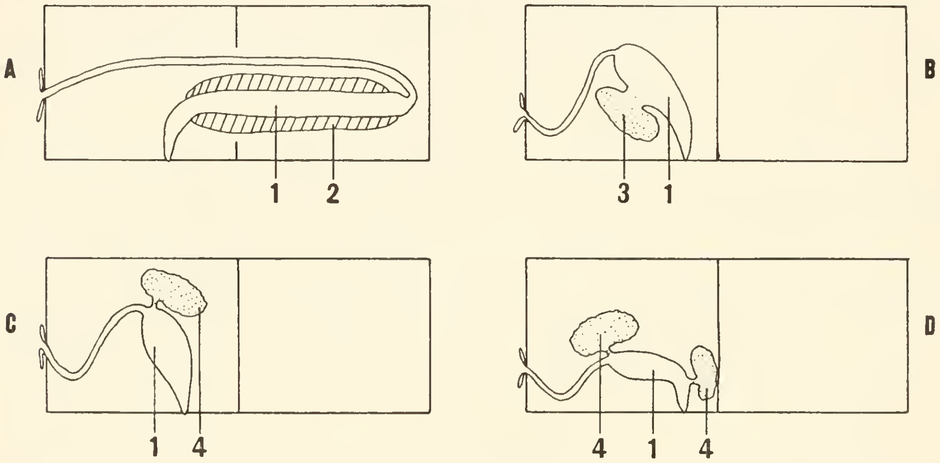

FIGURE 1. Diagrammatical representation of the male genitalia of A. *Clitellio;* B. *Limnodriloides;* C. *Spiridion;* D. *Phallodrilus.* 1. Atrium; 2. Diffuse prostate, absent in *C. arenarius;* 3. Discrete prostate, thickly-stalked; 4. Discrete prostate, pedunculate.

SPECIES EXAMINED FROM CAPE COD BAY

(?) *Tubifex longipenis* Brinkhurst, 1965. Southwest sector,[2], 7 to 18 meters depth. The three individuals attributed to this species from the Cape Cod Bay material were of similar size and setal pattern to the type specimens. However, they were immature and thus their identity must remain uncertain until mature material is available.

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[2] In this list "sector" refers to an approximate distribution of species on the following basis: Cape Cod Bay is considered as roughly circular in area; the four sectors correspond to the areas enclosed by bisecting this circle from north to south, and from east to west at 41°54′ N, 70°17′ W.

Peloscolex benedeni (Udekem, 1855). Southwestern sector, 7 to 18 meters depth. This species seems to be confined to shallow waters and is locally very abundant.

Peloscolex intermedius nov. sp. The two Northern, and Southwestern sectors, 7 to 46 meters depth.

Adelodrilus anisosetosus nov. gen., nov. sp. Southeastern sector, 18 meters depth.

Phallodrilus obscurus nov. sp. Southwestern sector, 8.5 meters depth.

Phallodrilus coeloprostatus nov. sp. Southeastern sector, 18 meters depth.

Limnodriloides medioporus nov. sp. The two Southern, and Northeastern sectors, 7 to 46 meters depth. This entity, and *P. intermedius,* are the dominant Tubificidae in Cape Cod Bay below 30 meters depth.

Systematic Section

Peloscolex intermedius nov. sp.

Figure 2

Holotype. United States National Museum (USNM) Cat. No. 38259. Cape Cod Bay, Massachusetts, USA. 41°55.75′ N, 70°21.07′ W. Depth 42.6 meters.

Paratypes. USNM 38260. Six individuals as type locality; 38261 one individual from 42°0.5′ N, 70°24′ W. Depth 36.5 meters; 38262 one individual from 41°55.4′ N, 70°15.9′ W, depth 42.6 meters.

Derivation. "Intermediate" between two other *Peloscolex* species.

Description. Length 8 to 10 mm, diameter 0.28 to 0.45 mm. About 42 segments. Prostomium small, conical, with small papilla anteriorly. Body wall smooth to densely, but very finely, granulate. Dorsal setae; from segment II to VI (sometimes VII) 3 bifids and 1 to 3 hairs per bundle present; bifids 50 to 80 μ long with equal teeth which become increasingly shorter in more posterior segments, or whose upper tooth becomes increasingly reduced; hairs 110 to 160 μ long, bearing a few very short, indistinct, lateral hairs; from segment VII (sometimes VIII) to the terminal segment, 3 simple-pointed, hair-like setae, 75 to 100 μ long plus 3 true hair setae, 110 to 160 μ long per bundle present. Ventral setae; anteriorly 3 to 4 per bundle, posteriorly 2 to 3 per bundle; ventral bifids, 60 to 80 μ long, with thin upper tooth and broad widely diverging lower tooth (Fig. 2b). One unmodified ventral seta per bundle on segments X and XI. One pair male and spermathecal pores situated just anterior to, and in line with ventral setae.

Looped to coiled vasa deferentia, 15 μ diameter, 1.0 mm long, join atria subapically and dorsally. Vasa deferentia 5 to 7 times longer than atria. Atria elongate, with long axis directed posteriorly and with muscle bands arranged circularly. Atria 150 to 220 μ long, 60 to 90 μ wide, joined to cylindrical, cuticularized penes, 75 to 100 μ long, 31 to 37 μ wide, by short, discrete proximal ducts. Large, discrete prostate glands join atria subapically to medially and ventrally (Fig. 2a). Spermathecae with sacciform ampullae and long discrete ducts which have a bulbous swelling proximally. Spermatophores spindle-shaped, 130 μ long, 35 μ diameter at the median swelling.

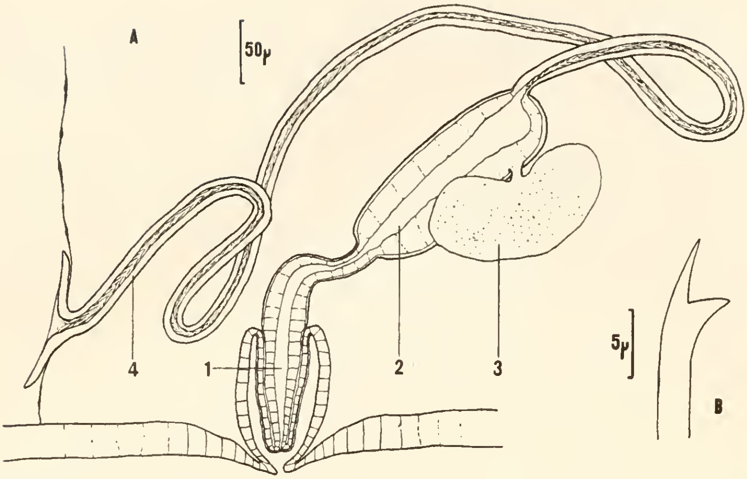

FIGURE 2. *Peloscolex intermedius* nov. sp. A. Male genitalia; B. Posterior ventral seta. 1. Cuticularized penis; 2. Atrium; 3. Prostate; 4. Vas deferens.

DISTRIBUTION. Known only from Cape Cod Bay, Massachusetts, in waters 7 to 46 meters deep.

REMARKS. *P. intermedius* is closely related to *P. apectinatus* Brinkhurst, 1965 and *P. swirencowi* (Jaroschenko, 1948). (Table 1.) These three species, together with *P. euxinicus* Hrabě, 1966, *P. gabriellae* Marcus, 1950 and *P. nerthoides* Brinkhurst, 1965, form a complex of small, sparsely or finely papillate, marine worms whose male genitalia bear a strong resemblance to those of *Tubifex*. It is suspected that future work on this group of species may reveal more intermediate forms in the complex which will invalidate many of the entities or reduce them to subspecific rank, and that such work will make necessary a critical reexamination of the generic limits of *Peloscolex* and *Tubifex*.

TABLE I

Differences between Peloscolex intermedius *nov. sp. and its two most closely related species,* P. apectinatus *and* P. swirencowi

Character	*P. intermedius*	*P. apectinatus*	*P. swirencowi*
Hair setae	With sparse lateral hairs	Serrate	Smooth
Anterior dorsal setae	Bifid to seg. VI	Bifid	Bifid to seg. VIII
Posterior dorsal setae	Single-pointed, elongate	Bifid	Single-pointed, elongate
Ventral setae; upper tooth compared to lower	Longer	Same or shorter	Longer
Length vas deferens/length atrium	4.5	less than 3	2.5
Length penis/diameter penis	2.4–2.6	1.5–1.6	1.0

Adelodrilus nov. gen.

Derivation. "Adelo-" = Gr. hidden/secret; "drilus" = worm.

Definition. Hair setae absent. Penial setae highly modified. Male and spermathecal pores paired in line of ventral setae.

Vasa deferentia very short, about 0.2 the length of the atria, join the latter apically. Atria thin walled, cylindrical, without connection with prostate cells, terminating in pear-shaped penial bulbs. Penial bulbs each bear two large, thickly-stalked, prostate glands. Spermatophores not developed. Coelomocytes small and only sparsely distributed.

Type-species. *Adelodrilus anisosetosus* nov. sp. by monotypy.

Adelodrilus anisosetosus nov. sp.

Figure 3

Holotype. USNM 38251. Cape Cod Bay, Massachusetts, USA. 41°53.5′ N, 70°10.65′ W. Depth 18.3 meters.

Paratypes. USNM 38252. Six individuals; locality as for Holotype.

Derivation. "Aniso-" = Gr. unequal; "setosus" = setae.

Description. Length 4 to 6.5 mm, diameter 0.18 to 0.4 mm. 30 to 45 segments. Prostomium broadly rounded, longer than it is wide at peristomium. Clitellum well developed on segments X to XII. Segments, especially in posterior part of body, deeply annulated with body wall nuclei concentrated in rows on the crests of the ridges formed by annuli. Annuli impart granular to papillate appearance to body wall. Setae 3 sometimes 4, per bundle in all body regions. Anterior, and posterior ventral, setae are bifid with widely diverging teeth, the upper of which become thinner and shorter in more posterior segments (Fig. 3b, c). Posterior dorsal setae single-pointed and strongly curved distally, 85 to 110 μ long (Fig. 3d). Anterior setae 80 to 100 μ long and mid body setae 70 to 75 μ long. Ventral setae of segment XI highly modified into penial bundles, each of which contains one giant, simple-pointed, strongly curved seta, 135 to 150 μ long, 6.5 to 8.8 μ thick, and 8 to 12 small, thin, straight setae, 70 to 90 μ long, 1.5 μ thick, which are clubbed distally and which bear a thin, hooked tooth, originating apically and curving around the club (Fig. 3e, f). Male and spermathecal pores paired in line of ventral setae.

Pharyngeal glands extend into segment VI. Male ducts consist of a pair of vasa deferentia, 40 μ long, 15 μ diameter, which join a pair of cylindrical, thin-walled atrial ampullae apically. Each atrial ampulla, 250 μ long, 28 to 37 μ diameter, joins a pear-shaped penial bulb. This structure, 50 to 65 μ long, 30 to 45 μ diameter, bears two thickly-stalked, discrete prostate glands, one apically near atrial junction, and one near the proximal end. Penial bulbs open into a pair of spherical pedunculate chambers about 70 μ diameter, into which also protrude the penial setae

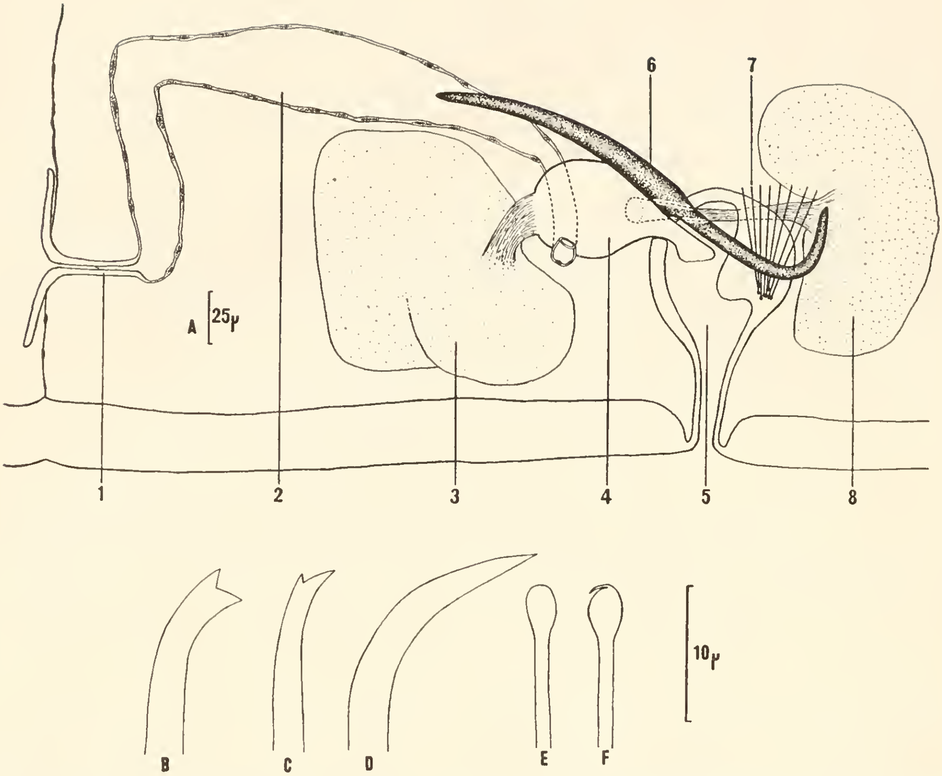

Figure 3. *Adelodrilus anisosetosus* nov. gen., nov. sp. A. Male genitalia; B. Anterior seta; C. Posterior ventral seta; D. Posterior dorsal seta; E, F. Small straight penial setae showing two aspects of distal club. 1. Vas deferens; 2. Atrium; 3. Anterior prostate; 4. Penial bulb; 5. Penial chamber; 6. Giant penial seta; 7. Small straight penial seta; 8. Posterior prostate.

(Fig. 3a). Penes are formed from the protruded ends of elongate lining cells of the penial bulb. Spermathecae with sacciform ampullae 200 µ long, up to 55 µ wide, and ill-defined ducts, 45 µ long, 25 µ diameter which open near septum IX/X.

Distribution. Known only from type-locality.

Remarks. The homologies of the male ducts of *Adelodrilus anisosetosus* are difficult to interpret. Thus the penial bulb (Fig. 3) is probably not equivalent morphologically to this structure in other Tubificidae. Morphogenetic studies are clearly necessary on this and on some related genera (see below) to clarify these anatomical details. From the functional point of view, however, this entity is unique among the tubificids in possessing prostate glands which are associated with a penial structure and in having two distinct modifications of the penial setae. The structure of the penis itself is also interesting in that it is formed from the internal lining cells of the penial bulb which are thought to become elongate at copulation. Such a penial structure has only been reported in the Lumbriculidae (Cook, 1967) and the Dorydrilidae (Cook, 1967; 1968).

It is possible that *Adelodrilus* is phylogenetically important as it may elucidate the nature and origin of the bulbous "paratria" found in *Bothrioneurum* Stolc, 1888 and *Smithsonidrilus* Brinkhurst, 1966 (Fig. 4). In both of these genera a cylindrical atrium and a bulbous "paratrium" enter a common chamber. It seems possible that in *Smithsonidrilus* the secretory and the storage functions of the male genital apparatus have become morphologically separated and that *Adelodrilus* is close to the ancestral form from which it and *Bothrioneurum* diverged. As a corollary to this hypothesis, the intromittent function of the penis must have become relocated in the storage part of the atria, or that this function became redundant by virtue of the presence of an eversible chamber acting as a pseudopenis in the sense of Brinkhurst (1965a).

Phallodrilus Pierantoni, 1902

Definition. Hair setae absent. Ventral setae of segment XI usually modified. Male pores paired, near ventral setae. Spermathecal pores paired, lateral to near ventral setae, or unpaired, mid dorsal.

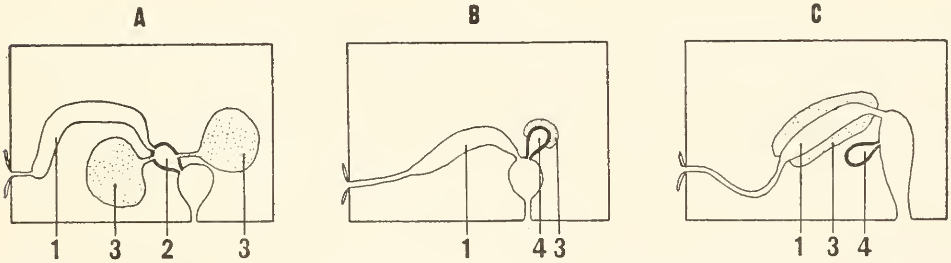

Figure 4. Diagrammatic representation of the male genitalia of A. *Adelodrilus;* B. *Smithsonidrilus;* C. *Bothrioneurum.* 1. Atrium; 2. Penial bulb; 3. Prostate; 4. "Paratrium."

Vasa deferentia as long as, or slightly longer than pear-shaped to cylindrical atria. Vasa deferentia join atria apically. Each atrium bears two discrete, pedunculate prostate glands, one of which joins near the vas deferens, the other near the proximal end of the atrium. Spermatophores not developed. Coelomocytes sparse to absent.

Type species: *Phallodrilus parthenopaeus* Pierantoni, 1902.

Remarks. As defined above, *Phallodrilus* includes *Aktedrilus* Knollner, 1935. The only character which has been used to keep these two genera separate in the past has been that *Aktedrilus* possesses only a single, dorsal spermatheca. However, this separation is considered to be invalid due to the fact that in *Phallodrilus coeloprostatus* the spermathecal pores are more dorsal than ventral, and that in some other genera paired versus unpaired spermathecae are clearly specific characters (*e.g., Monopylephorus, Eclipidrilus* (Lumbriculidae)).

KEY TO SPECIES.

1. Penial setae absent. Spermatheca unpaired, with mid-dorsal pore.......... ....*P. monospermathecus* (Knollner, 1935)
Penial setae present. Spermathecae paired, with lateral to ventral pores....2
2(1). Up to 13 hooked penial setae, shorter than body setae, present. Spermathecal pores situated between lines of dorsal and ventral setae..*P. coeloprostatus* nov. sp.
Up to 7 bifid to simple-pointed penial setae, longer than body setae, present. Spermathecal pores situated near to ventral setae.............3
3(2). Spermathecal setae with upper teeth 2.5 times longer than lower teeth..............................*P. parthenopaeus* Pierantoni, 1902.
No modified spermathecal setae...4
4(3). 4 to 6 (rarely 7) unmodified to single-pointed penial setae present. Spermathecal ducts short and narrow.........*P. aquaedulcis* Hrabě, 1960
2 to 3 (rarely 4) single-pointed penial setae present. Spermathecal ducts long and thick...........................*P. obscurus* nov. sp.

Phallodrilus coeloprostatus nov. sp.

Figure 5

HOLOTYPE. USNM 38257. Cape Cod Bay, Massachusetts, USA. 41°53.5′ N, 70°10.65′ W. Depth 18.3 meters.

PARATYPES. USNM 38258. Six individuals; locality as for Holotype.

DERIVATION. "Coelo-" = Gr. hollow; "prostatus" = prostate.

DESCRIPTION. Length 6 to 10 mm, diameter 0.17 to 0.30 mm. 58 to 60 segments. Prostomium rounded, as long as, or a little longer than it is broad at peristomium. Clitellum well developed on segments 1/2X to XII. Setae bifid with upper teeth shorter and thinner than lower, 48 to 55 μ long; 4 to 5 per bundle anteriorly, 3 to 4 posteriorly (Fig. 5c). Ventral setae of segment X unmodified. 10 to 13 penial setae, 40 to 45 μ long, hooked distally, present on segment XI (Fig. 5d). Paired male pores situated just lateral to penial setae. Paired spermathecal pores situated in anterior part of segment X, mid way between lines of dorsal and ventral setae.

Pharyngeal glands extend into segment VI. Chlorogogen cells begin in segment VI. Atria very small, cylindrical, curved towards anterior end of animal and very closely applied to body wall (Fig. 5a). Vasa deferentia, 6 μ diameter, longer than atria, join latter apically. Atria 95 to 130 μ long, 19 to 26 μ diameter, with very thin musculature and thick lining cells, terminating in small, truncated cone-shaped penes. Two pairs of very large prostate glands join atria by thick, discrete ducts, one near vasa deferentia, the other posteriorly, near the proximal end of the atria. Prostates, which lie medially to, and completely cover atria, have distinct boundaries, but the cells are loosely packed and cavities form between them (Fig. 5b). Paired spermathecae have short, discrete ducts and large ovoid to elongate ampullae.

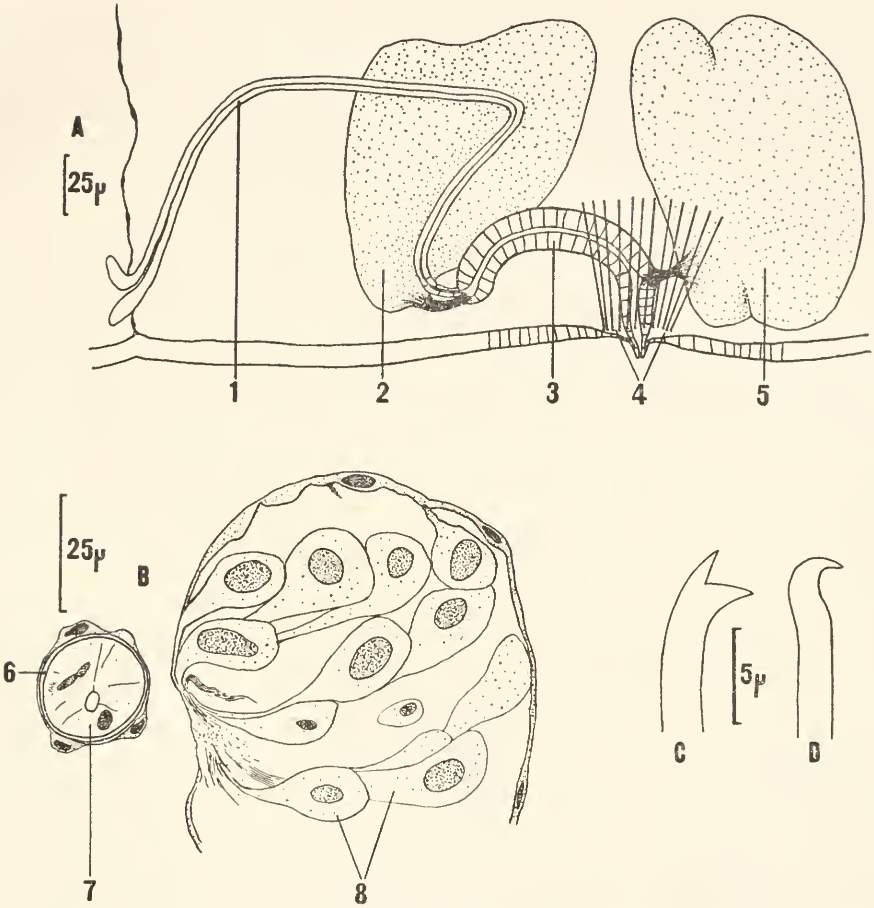

Figure 5. *Phallodrilus coeloprostatus* nov. sp. A. Male genitalia; B. Transverse section of atrium and prostate gland; C. Seta; D. Penial seta. 1. Vas deferens; 2. Anterior prostate; 3. Atrium; 4. Penial setae; 5. Posterior prostate; 6. Atrial wall; 7. Atrial lining cells; 8. Prostate cells.

Distribution. Known only from type-locality.

Phallodrilus obscurus nov. sp.

Figure 6

Holotype. USNM 38255. Cape Cod Bay, Massachusetts, USA. 41°51.0′ N, 70°31.1′ W. Depth 8.5 meters.

Paratypes. USNM 38256. Six individuals; locality as for Holotype.

Derivation. "Obscure" relationship to other species of the genus.

DESCRIPTION. Length 7 mm, diameter 0.16 to 0.20 mm. 40 segments. Prostomium longer than it is broad at peristomium. Setae, except ventrals of segment XI, bifid with upper tooth equal to, or shorter and thinner than lower tooth (Fig. 6b). Setae 45 to 55 μ long, 4 to 6 per bundle anteriorly, 4 to 5 per bundle posteriorly. Ventral setae of segment X unmodified. 2 to 3 (rarely 4) slightly curved, simple-pointed penial setae, 55 to 70 μ long, present (Fig. 6c). One pair spermathecal pores in anterior part of segment X, in line with ventral setae. Male pores just lateral to penial setae.

Pharyngeal glands extend into segment V. Chlorogogen cells begin in segment VI. Atria pear-to-comma-shaped, shorter than vasa deferentia which join atria apically. Vasa deferentia 100 to 130 μ long, 5.5 to 7.5 μ diameter. Atria 70 to 120 μ long, 30 to 35 μ diameter. Anterior prostate gland enters atrium near vas deferens, posterior one 10 to 40 μ from the male pore (Fig. 6a). Penes absent. One pair spermathecae with long, thick ducts and cylindrical ampullae, open near septum IX/X.

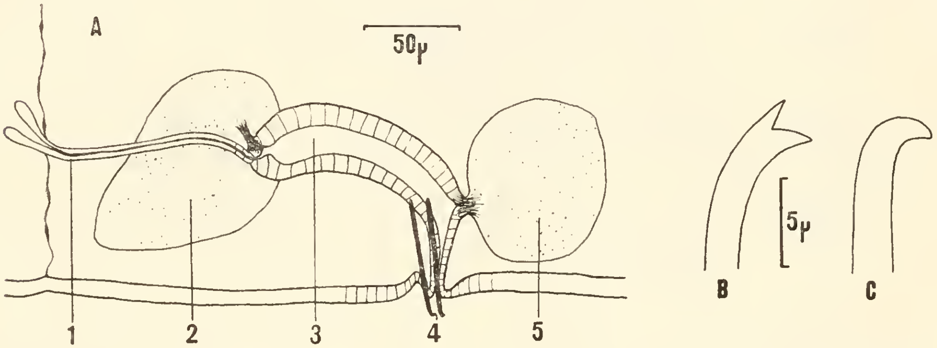

FIGURE 6. *Phallodrilus obscurus* nov. sp. A. Male genitalia; B. Seta; C. Penial seta. Explanations as in Figure 5.

DISTRIBUTION. Known only from type-locality.

REMARKS. *P. obscurus* is very closely related to *P. parthenopaeus* and *P. aquaedulcis.* These three entities form a complex whose taxonomic rank is uncertain. It is possible that they are subspecies or merely intra-specific variants or differing stages of maturation.

Phallodrilus parthenopaeus Pierantoni, 1902

Phallodrilus parthenopaeus Pierantoni, 1903, pp. 114, 115, fig. 1, 2: Hrabě, 1960, p. 251: Brinkhurst, 1963a, p. 74; 1963b, p. 714; 1967, p. 115.

TYPE-MATERIAL. Not designated and not located. Type-locality Gulf of Naples, Italy. Depth 4 meters.

Description, (from literature). Length up to 12 mm, diameter 0.2 mm. 40 to 60 segments. Setae, except ventral of segments X and XI, bifid with equal teeth. 4 setae per bundle anteriorly, 2 per bundle posteriorly. 2 spermathecal setae per bundle with upper teeth about 2.5 times longer than lower. 2 straight, blade-shaped penial setae per bundle, with simple, rounded ends. Male and spermathecal pores paired, near line of ventral setae.

Atria elongate pear-shaped, shorter than vasa deferentia which join atria apically. Anterior prostate gland opens near vas deferens, posterior one near base of atrium. Penes absent. Spermathecae paired in segment X.

Distribution. Known only from type-locality.

Phallodrilus aquaedulcis Hrabě, 1960

Phallodrilus aquaedulcis Hrabě, 1960, pp. 248–251, fig. 1–4: Brinkhurst, 1963a, pp. 74, 77.

Type-material. Hrabě collection; catalogue numbers not designated. Locality, Blumenthal, River Weser, Germany. In fresh-water.

Description, (from literature). Length 3 to 4 mm, diameter 0.15 mm. 28 to 32 segments. Prostomium rounded, as long as it is broad at peristomium. Setae, except ventrals of segment XI, bifid with upper tooth thinner and shorter than lower, about 42 μ long. 3 to 4 (rarely 5) setae per bundle anteriorly, 2 per bundle posteriorly. Ventral setae of segment X unmodified. 4 to 6 (rarely 7) penial setae present; these unmodified bifids to single-pointed setae. Spermathecal pores in anterior part of segment X, in line with ventral setae. Male pores slightly anterio-lateral to penial setae.

Pharyngeal glands present in segments III to V. Chlorogogen cells begin in segment V or VI. Atria cylindrical, longer than vasa deferentia which open into atria apically. Anterior prostate gland opens near vas deferens, posterior one near proximal part of atrium. Penes absent. Spermathecae paired, with long, cylindrical ampullae and short, narrow ducts.

Distribution. Known only from type-locality.

Phallodrilus monospermathecus (Knollner, 1935) nov. comb.

Aktedrilus monospermathecus Knollner, 1935, pp. 482–491, fig. 43–50: Bulow, 1955, p. 262; 1957, p. 102: Hrabě, 1960, pp. 251–254, fig. 5–12: Cekanovskaya, 1962, p. 286: Brinkhurst, 1963a, p. 75; 1963b, p. 714; 1963c, p. 1203; 1964, p. 12; 1967, p. 115.

Type-material. Not designated and not located. Type-locality, Kiel Bay, West Germany, Marine, littoral.

Other material. R. O. Brinkhurst collection; many individuals from saline moat, Hale, Lancashire, England.

Description, (from literature, some characters confirmed by author). Length 3 to 8 mm, diameter 0.11 to 0.23 mm. 25 to 35 segments. Prostomium longer than it is broad at peristomium. Setae bifid with upper tooth shorter and thinner than lower, 32 to 45 μ long. 3 to 4 (rarely 2 to 5) setae per bundle anteriorly, 2 to 3 (rarely 1 to 4) per bundle posteriorly. Ventral setae of segment XI absent. Spermathecal pore single, mid-dorsal on segment X; cuticle thickened in region of pore. Male pores paired in line of ventral setae.

Pharyngeal glands in segments IV to VI. Chlorogogen cells begin in segment VI. Atria narrow, cylindrical, about as long as vasa deferentia. Atria, 16 to 17 μ diameter, terminate in ovoid penes 25 to 30 μ long, 17 to 19 μ wide, contained in penial chambers. Anterior prostate glands open into atria apically with vasa deferentia, smaller posterior prostates enter near proximal end of atria. Spermatheca single, cylindrical, with thick duct opening mid-dorsally.

Distribution. Mainly intertidal zone of the Baltic Sea, Mediterranean Sea and Northeastern Atlantic. Also brackish-water and ground-water.

Limnodriloides Pierantoni, 1903

Definition. Hair setae absent. Ventral setae of segment XI unmodified. Male and spermathecal pores paired, more or less in line with ventral setae, or contained within a large, common, mid-ventral fold.

Gut in immediate preclitellar region with a pair of elongate diverticulae. Vasa deferentia, as long as or slightly longer than atria, join latter more or less apically. Each atrium bears a discrete prostate gland, broadly attached ventral or anterior to vas deferens. Atria with thin muscle layer. Penes present or absent. Spermatophores absent but sperm often aggregates into more or less discrete, oriented bundles. Coelomocytes absent.

Type-species. *Limnodriloides appendiculatus* Pierantoni, 1903.

Remarks. *Limnodriloides* was included in *Clitellio* by Brinkhurst (1963a) but was reinstated by Hrabě (1967) who included *Thallassodrilus prostatus* (Knollner, 1935) within it. *Limnodriloides* is considered to be distinct from *Clitellio* by virtue of three contrasting criteria, thus: *Limnodriloides* has 1) gut diverticulae, 2) broadly attached, discrete prostate glands, and 3) no spermatophores while *Clitellio* possesses 1) no gut diverticulae, 2) no prostate gland or a diffuse one, and 3) well developed spermatophores (see also Fig. 1). *T. prostatus* (Knollner) is excluded from *Limnodriloides* as it has no gut diverticulae, possesses a series of penial setae, and has a peculiarly thick muscular atrium with a pedunculate prostate gland.

In his original description of the genus, Pierantoni (1904) included the species *L. roseus* and *L. pectinatus* in *Limnodriloides*. These two species have no gut diverticulae, the former have pedunculate prostate glands joining the atria dorsal or posterior to the vasa deferentia, and the latter possesses a series of modified penial setae. Neither species has apparently been seen by other workers who have regarded them as *species dubiae* of *Limnodriloides*. Since *Spiridion* Knollner, 1935, has penial setae and pedunculate prostate glands which join the atria dorsal or posterior to the vasa deferentia, it is proposed that *L. roseus* and *L. pectinatus* should be included as *species dubiae* of this genus. This action clarifies the definitions of this group of marine genera as *Limnodriloides,* excluding *L. roseus* and *L. pectinatus*, becomes a clearly homogeneous group, while *Spiridion,* even with its new *species dubiae,* retains its cohesion as a genus.

KEY TO SPECIES.

1. Male and spermathecal pores contained within a median ventral fold in body wall; *i.e.,* external apertures appear as elongate median slits arranged transversely .... *L. medioporus* nov. sp.
Male and spermathecal pores paired, in line of ventral setae; *i.e.,* external apertures appear as simple, paired pores 2
2(1). Ventral setae of segment X modified and contained within a muscular sac .... *L. winckelmanni* Michaelsen, 1914
Ventral setae of segment X unmodified or absent 3
3(2). Gut diverticulae in segment VIII. 2 setae per bundle in most posterior segments .... *L. appendiculatus* Pierantoni, 1903
Gut diverticulae in segment IX. 1 seta per bundle in most posterior segments .... *L. agnes* Hrabě, 1966

Limnodriloides medioporus nov. sp.

Figure 7

HOLOTYPE. USNM 38253. Cape Cod Bay, Massachusetts, USA. 41°54.9′ N, 70°15.2′ W. Depth 36.5 meters.

PARATYPES. USNM 38254. Seven individuals; locality as for Holotype.

OTHER MATERIAL. Author's collection, from Woods Hole Oceanographic Institution's Gay Head—Bermuda transect: 40°20.5′ N, 70°47′ W, depth 97 meters (8 individuals).

DERIVATION. "Medio-" = L. middle; "porus" = pore/hole.

DESCRIPTION. Length 8 mm, diameter 0.2 mm. 40 segments. Prostomium usually longer than it is broad at peristomium, with a small, thin-walled papilla on its tip. Setae 2 to 4 per bundle anteriorly, 2 per bundle posteriorly. Setae bifid with teeth of about equal length, 30 to 50 μ long (Fig. 7c). Ventral setae absent on segments X and XI. Clitellum on segments IX to XII. One pair of spermathecal pores open ventral to line of ventral setae and are joined by a laterally elongated, median invagination of the body wall of segment X. One pair male pores open inside a dumbell-shaped, median bursa on segment XI.

Pharyngeal glands penetrate into segment V. Chlorogogen cells begin in segment VI. A pair of diverticulae present on the gut, joining this in the posterior part of segment IX and extending anteriorly to septum VIII/IX. Vasa deferentia, 11 to 14 μ diameter and as long as, or slightly shorter than atria, join latter apically. Atria cylindrical, 110 to 130 μ long, 40 to 55 μ diameter, which narrow to a pair of ducts, 70 to 80 μ long, (relaxed condition), 15 μ diameter. These ducts terminate

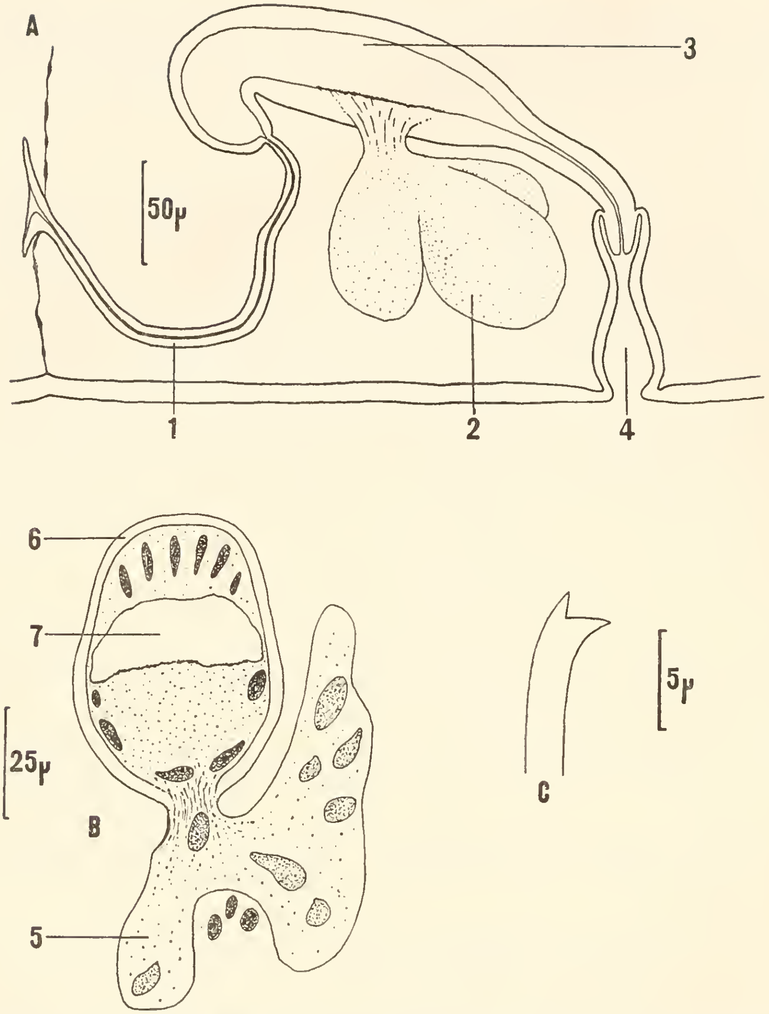

Figure 7. *Limnodriloides medioporus* nov. sp. A. Male genitalia; B. Transverse section of atrium and prostate gland; C. Seta. 1. Vas deferens; 2. Prostate; 3. Atrium; 4. Median genital chamber; 5. Prostate cells; 6. Dorsal atrium wall; 7. Atrial lumen.

in small, conical penes, 25 μ long, which open into a median chamber, mediolaterally (Fig. 7a). Long axis of atria directed anteriorly. Large compact prostate glands open into atria medially and ventrally. Lining cells of ventral part of atria thicker than dorsal cells (Fig. 7b). One pair spermathecae with ovoid ampullae and short, ill-defined ducts, present in segment X. Ducts open into common median chamber. Sperm in spermathecae, often oriented in a definite manner and in discrete masses.

Distribution. Continental shelf, Massachusetts, USA.

Limnodriloides appendiculatus Pierantoni, 1903

Limnodriloides appendiculatus Pierantoni, 1904, pp. 187–188, fig. 1: Boldt, 1928, pp. 145–151, fig. 2–3: Hrabě, 1967, pp. 339, 344.
Clitellio appendiculatus (Pierantoni). Brinkhurst, 1963a, p. 73; 1963b, p. 713; 1966, p. 300; 1967, p. 115.

Type-material. Not designated and not located. Type-locality; Gulf of Naples, Italy. Depth 3 meters.

Description, (from literature). Length 10 to 18 mm, diameter 0.2 to 0.4 mm. 40 to 50 segments. Clitellum on segments 1/2X to 1/2XII. Setae bifid with upper tooth thinner, and posteriorly shorter, than lower. 2 (rarely 3) setae per bundle anteriorly and 2 posteriorly. No modified genital setae. Male and spermathecal pores situated just anterior to ventral setae of segments X and XI, respectively.

Pharyngeal glands present in segments III to V. Chlorogogen cells begin in segment VI. A pair of intestinal diverticulae present in segment VIII, extending to septum VII/VIII. Vasa deferentia, 13 μ diameter, as long as, or slightly shorter than atria which they join apically. Atria cylindrical, with a median constriction and narrowing proximally. Atria open into a pair of pear-shaped, eversible pseudopenes laterally. Large, discrete, broadly-attached prostate glands join atria subapically. Paired spermathecae in segment X with very short, ill-defined ducts.

Distribution. Known only from type-locality.

Limnodriloides agnes Hrabě, 1966

Limnodriloides agnes Hrabě, 1967, pp. 339–344, fig. 13–24.

Type-material. Syntypes (?) Hrabě collection No. 1766—1, 4. Nesebar (Mesembria), near Fishermans Pier, Black Sea, Bulgaria.

Description, (from literature). Length 10 mm, diameter 0.4 mm. 50 to 68 segments. Prostomium rounded. Clitellum developed on segments 1/2X to XII. Setae bifid with upper tooth shorter and thinner than lower, 2 (rarely 3) per bundle anteriorly, 1 per bundle posteriorly, 77 to 112 μ long. Ventral setae of segment X unmodified, of segment XI absent. Paired spermathecal pores anterior, and a little lateral to ventral setae of segment X. Paired male pores in place of ventral setae of segment XI.

Pharyngeal glands in segments III to IV. Chlorogogen cells begin in segment VI. A pair of intestinal diverticulae present in segment IX which extend to septum VIII/IX. Vasa deferentia shorter than atria which they join apically. Diameter of vasa deferentia near small male funnels, 16 μ widening to 25 μ near atrial junction. Atria elongate and tapering proximally, 38 μ diameter near vasa deferentia, narrowing to 11 μ about half way along its length. Atria open into a pair of large, eversible pseudopenes, 208 μ long, 64 μ diameter. Large, compact, non-pedunculate prostate glands join atria near vasa deferentia. Paired spermathecae with long, cylindrical ampullae and very short, inconspicuous ducts.

Distribution. Known only from type-locality.

Limnodriloides winckelmanni Michaelsen, 1914

Limnodriloides winckelmanni Michaelsen, 1914, pp. 155–160, Pl. V, fig. 6, 7: Boldt, 1928, pp. 146–148, fig. 1: Hrabě, 1967, pp. 339, 345–347, fig. 25–29.
Clitellio winckelmanni (Michaelsen). Brinkhurst, 1963a, p. 73; 1963b, p. 713; 1966, p. 153.

Type-material. Not designated and not yet located. Type-locality, Swakopmund, South West Africa. Intertidal, under stones.

Description, (from literature). Length 12 to 18 mm, diameter 0.2 to 0.25 mm posteriorly and 0.6 mm in clitellar region. 3 setae per bundle anteriorly, 2 per bundle posteriorly, smaller than middle setae. Middle setae 90 μ long, 5 μ thick with lower tooth 5 μ long. 1 ventral modified seta per bundle of segment X; this spermathecal seta hollow, contained in large vacuolated gland cells and surrounded by thick muscle layer. Male and spermathecal pores paired in line of ventral setae.

Paired intestinal diverticulae present in segment IX. Vasa deferentia as long as atria, join latter apically. Atria ovoid with long, narrow, proximal ducts, terminating in small penes (Hrabě, 1967, states that it has no penes but illustrates a penis-like structure). Large prostate gland joins each atrium ventrally on a broad base. Spermathecal ampullae sacciform, with thick, discrete ducts. Sperm oriented into long, narrow bundles.

Distribution. Known only from type-locality.

Spiridion Knollner, 1935

Definition. Hair setae absent. Ventral setae of segment XI modified into a row of penial setae. Male and spermathecal pores paired, more or less in line of ventral setae.

Vasa deferentia about as long as atria. One discrete, pedunculate prostate gland joins each atrium almost apically but dorsal or posterior to junction of vas deferens. Atrial muscle thin. True penes absent. Spermatophores not developed. Coelomocytes absent.

Remarks. No key to species is provided as three out of the four species here attributed to *Spiridion* are *species dubiae*.

Type species: *Spiridion insigne* Knollner, 1935.

Spiridion insigne Knollner, 1935

Spiridion insigne Knollner, 1935, pp. 427, 471–475, fig. 35–38: Bulow, 1957, p. 98: Hrabě, 1960, p. 255, fig. 13–14: Cekanovskaya, 1962, p. 243: Brinkhurst, 1963a, pp. 74, 75, fig. 2, 57; 1967, p. 115.
Spiridon insigne Knollner. Brinkhurst, 1963b, pp. 712, 714.
(?) *Spiridion insigne* Knollner. Brinkhurst, 1965b, p. 153.

Type-material. Not designated and not located. Type-locality, Strander Bach, Schilkseebucht, Kiel Bay, Baltic Sea, West Germany.

Description, (from literature). Length 5 to 10 mm, diameter 0.34 mm at segment XI, 0.13 mm diameter in pre-clitellar segments. 34 segments. Setae bifid with upper tooth shorter and thinner than lower, 35 to 45 μ long. 3 to 5 setae per bundle anteriorly, 1 to 2 (sometimes 3) posteriorly. 4 to 6 single-pointed, hooked penial setae, 77 to 90 μ long. Ventral setae of segment X absent. Male pores anterior to and in line with penial setae. Spermathecal pores, in line with ventral setae, present in anterior part of segment X.

Vasa deferentia a little longer than atria, join latter apically. Atria cylindrical, 140 μ long, 20 μ diameter. Prostate gland enters atrium near vas deferens. Spermathecae with long, cylindrical ampullae and short discrete ducts which open near septum IX/X.

Distribution. Marine littoral Baltic Sea. Ground-water, Germany. West Atlantic coast (?)

Species dubiae

Spiridion scrobicularae Lastockin, 1937

Spiridion scrobicularae Lastockin, 1937, p. 234.

Spiridion scrobiculare Lastockin. Brinkhurst, 1963a, p. 75; 1967, p. 115.

Type-material. Not designated and not located. Type-locality, Coparskaya Bay, Gulf of Finland, USSR.

Description. Clitellum rudimentary, developed only in region of spermathecal pores and genital cavity. Male pore unpaired, median. Penial setae present. Paired atria muscular, consisting of a narrow anterior part and pear-shaped posterior part. Prostate gland joins atrium apically. Both atria and bundles of penial setae open into the median genital chamber.

Distribution. Type-locality only.

Spiridion roseus (Pierantoni, 1903) nov. comb.

Limnodriloides roseus Pierantoni, 1904, pp. 188, 189, fig. 2: Boldt, 1928, pp. 146–148: Hrabě, 1967, p. 347.
Clitellio roseus (Pierantoni). Brinkhurst, 1963a, p. 73; 1963b, p. 713.

Type-material. Not designated and not located. Type-locality, Gulf of Naples, Italy. Depth 3 to 4 meters.

Description. Setae bifid, 4 per bundle anteriorly, 3 per bundle posteriorly. Vasa deferentia short, join atria apically. Atria elongate, pear-shaped to cylindrical, erect, terminating in short protrusible penes. Large prostate gland joins each atrium just posterior to junction of vas deferens.

Distribution. Type-locality only.

Spiridion pectinatus (Pierantoni, 1903) nov. comb.

Limnodriloides pectinatus Pierantoni, 1904, pp. 190, 191, fig. 3: Boldt, 1928, pp. 146–148: Hrabě, 1967, p. 347.
Clitellio pectinatus (Pierantoni). Brinkhurst, 1963a, p. 73; 1963b, p. 713.

Type-material. Not designated and not located. Type-locality, Gulf of Naples, Italy.

Description. Length 12 to 15 mm, diameter 0.25 mm. 50 segments. Clitellum on segments 1/2X to 1/2XII. Setae bifid, 4 per bundle up to about segment XIV, then 2 to 3 per bundle. Ventral setae of segment XI modified to 12 small penial setae situated on tubercles. Paired male pores lateral to penial setae. Spermathecal pores in line with and anterior to ventral setae. Short vasa deferentia and large prostate glands join atria apically.
Distribution. Type-locality only.

Discussion

In the generic definition of *Phallodrilus,* coelomocytes were said to be "sparse to absent." *P. coeloprostatus,* for example, possesses a few small, free, darkly-staining cells in the coelom. In *Adelodrilus* the situation is similar. Hrabě (1963; 1966; 1967) has used the presence or absence of coelomocytes as the major character separating his subfamilies Tubificinae and Rhyacodrilinae. Clearly the intermediate condition of *Phallodrilus* and *Adelodrilus* invalidates the erection of subfamilies of Tubificidae on the basis of the presence or absence of coelomocytes.

My gratitude goes to Dr. R. O. Brinkhurst (University of Toronto) and Dr. R. P. Higgins (Systematics-Ecology Program, Marine Biological Laboratory) who offered useful criticism of this manuscript and to Dr. D. C. Grant (Systematics-Ecology Program) who made the Cape Cod Bay material available to me. My gratitude also to Dr. M. R. Carriker with whose program this project was carried out. The investigation was supported by grants from the Ford Foundation and from the National Science Foundation (GB-7387) to the Systematics-Ecology Program, Marine Biological Laboratory.

Summary

1. Marine Tubificidae from Cape Cod Bay, received from the Systematics-Ecology Program's Biotic Census, were examined.

2. One new genus and five new species are described, *Adelodrilus anisosetosus* nov. gen., nov. sp., *Peloscolex intermedius, Limnodriloides medioporus, Phallodrilus obscurus* and *Phallodrilus coeloprostatus.*

3. The genera *Limnodriloides, Phallodrilus* and *Spiridion* are reviewed.

4. *Aktedrilus* is made a junior synonym of *Phallodrilus.*

5. *L. roseus* Pierantoni, 1903 and *L. pectinatus* Pierantoni, 1903 are treated as *species dubiae* of *Spiridion.*

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OXYGEN CONSUMPTION OF TEMPERATURE-ACCLIMATED TOADFISH, *OPSANUS TAU*

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Temperature acclimation in poikilotherms has long been recognized in terms of its physiological consequences: in nature, by the ability of a poikilothermic species to maintain fairly constant activity over a range of habitat temperatures; in the laboratory, by the compensation of metabolism occurring in days or weeks after transfer to a new temperature (Bullock, 1955). The latter has generally been assayed by determinations of oxygen consumption, and a large number of cases have been reported (Bullock, 1955; Prosser, 1962). Among vertebrates, Kanungo and Prosser (1959) showed that acclimation to 10° compared with 30° increased the standard oxygen consumption of goldfish by 25% when measured at 20°, and 75% when measured at 25°. Roberts (1960) found that 5°-acclimated carp consume 60–70% more oxygen at 20° than 20°-acclimated animals. In sunfish, Roberts (1967) has shown that compensation of standard metabolism is almost complete for fish acclimated in the range of about 10° to 20°.

Previous studies in this laboratory have dealt with the role of the protein synthetic system in relation to the biochemical basis of temperature acclimation (Haschenmeyer, 1968; 1969a; 1969b). The toadfish, a marine fish of wide distribution, was used as the experimental animal in preference to other available species because of its adaptability to laboratory conditions and experimental procedures. Although oxygen consumption of toadfish at varying oxygen tensions has been reported (Hall, 1929), no data are available on the effect of temperature acclimation on the metabolism of this species. Therefore, a study has been made of oxygen consumption of toadfish under conditions of acclimation and handling identical to those used in the previous studies.

MATERIALS AND METHODS

Animals

Toadfish of intermediate sizes, 200–280 g, were obtained from the Supply Department at the Marine Biological Laboratory, Woods Hole. Control fish were kept in running sea water aquaria at 21° ± 1°, the temperature of the sea water supply. Cold-acclimated fish were kept for 9–12 days in similar aquaria maintained at 10° ± 1° using the refrigerated sea water supply at the Marine Biological Laboratory. They were starved throughout the acclimation period. Control fish were starved about 7 days. Before measurement of oxygen consumption at 22° ± 1°, cold-acclimated fish were transferred to 15° sea water and then to 21° sea water for 1–3 hours, to permit gradual adjustment to the temperature of measurement.

Oxygen measurement

A cylindrical lucite vessel, 6 liters volume, with ports for filling, air removal, and introduction of the electrode, was used as the experimental chamber. One end could be removed to introduce the animal and then sealed with a greased rubber gasket. A polarographic oxygen electrode designed by Kanwisher (1959, 1962) was used to continuously monitor the oxygen content of the sea water. Some characteristics of this type of electrode have been reported recently (Carey and Teal, 1965). An external magnet was used to rotate a small stirring bar at the electrode face (Kanwisher, 1959) and to agitate a second stirring bar suspended in a mesh bag which served to mix the contents of the chamber. The signal from the electrode was calibrated at the top of the scale with well aerated sea water at 22° and at the base line with water deaerated with nitrogen gas.

For each measurement the chamber was filled with fresh sea water, the fish was gently introduced, and the chamber sealed excluding air bubbles. The fish quickly settled on the sloping bottom of the chamber and generally did not move from this position throughout the measurement. The decrease in oxygen concentration in the

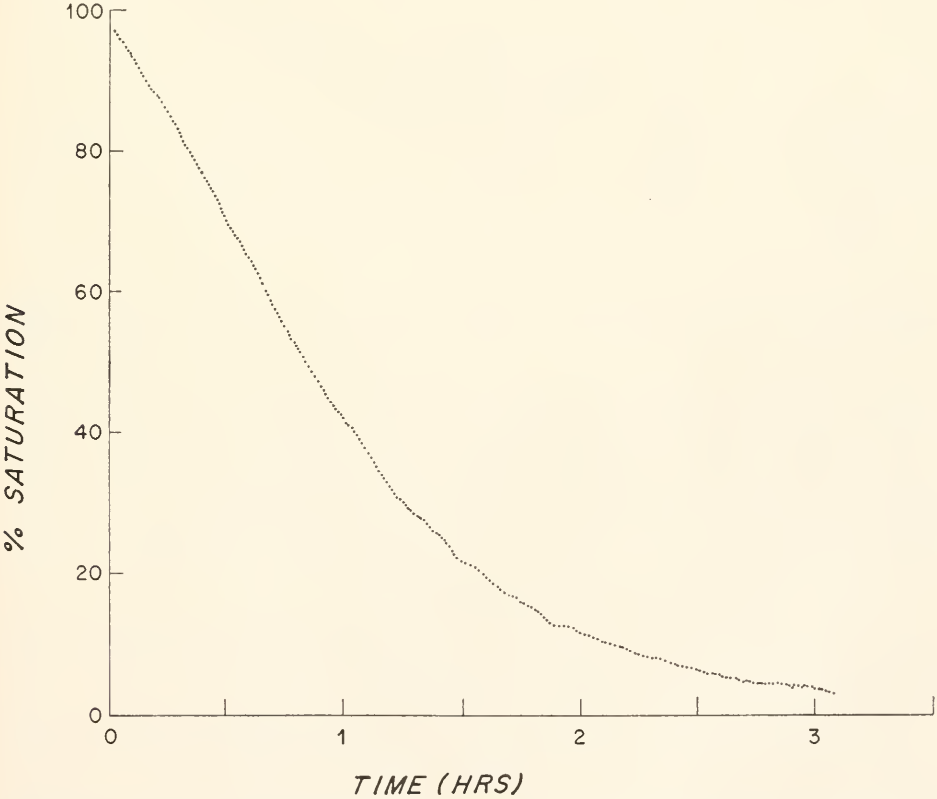

FIGURE 1. Polarographic oxygen electrode recording of oxygen consumption by a toadfish in a closed chamber.

chamber was followed for at least two hours, with oxygen values recorded automatically 75 times per minute.

Calculations of oxygen consumption were based on the linear decrease in per cent oxygen saturation in the range of 40–70% saturation. Because the toadfish is an oxygen conformer (Hall, 1929), it is necessary that all data be obtained in the same range of oxygen tensions. The observed values, ranging from 20% to 40% per hour, were corrected for a background decrease of 3% per hour observed in the absence of an experimental animal and were then converted to ml O_2/hr using the water volume of the chamber (5.9 l) and a value of 5.09 ml O_2/l for air-saturated sea water of salinity 32% at 22° and one atmosphere total pressure.

RESULTS

Figure 1 illustrates the results obtained from the continuous measurement of oxygen consumption of a toadfish in a closed respiratory chamber. The utilization of oxygen was essentially linear with time down to about 40% saturation; below this level the decay was exponential. A plot of log O_2 *vs.* time was linear in the range of 60% down to 3% saturation, indicating a first-order dependence of oxygen consumption on oxygen concentration. This observation is consistent with Hall's (1929) findings for the toadfish. At the very low oxygen levels reached at the end of some experiments, the fish showed stronger and more rapid opercular movements but appeared normal and unharmed when returned to aerated sea water.

TABLE I

Oxygen consumption of control (21°)-toadfish and 10°-acclimated toadfish at 22°

Acclimation temperature	Number of animals	Average weight (grams)	mlO_2/hr/kg	% increase
21°	4	264	24.4 ± 3.4 (S.D.)	—
10°	8	243	34.6 ± 6.2	42%

The collected results for oxygen consumption measured at 22° of toadfish acclimated to 10° or to 21° are given in Table 1. The cold-acclimated fish show 42% greater oxygen utilization than the control 21° group; analysis by the t-test indicates the difference between the means to be significant at $P = 0.01$. The small difference (9%) in average weights of the two groups is not sufficient to account for the observed difference in oxygen consumption. All of the toadfish remained quiet during transfer from one temperature to another and throughout the course of the oxygen measurement. Thus, there was no indication that the higher oxygen consumption of the cold-acclimated group was due to stress or activity. It may, therefore, be attributed to metabolic compensation.

In addition to the measurements at 22°, one fish from each acclimation group was measured in refrigerated sea water at a starting temperature of 12°. The chamber was partially insulated so that the temperature rose only 5° during the two hour period of the experiment. Under these conditions the control fish consumed about 10 ml O_2/hr/kg; the 10°-acclimated fish consumed about 20 ml

O_2/hr/kg. Although these results are not complete, they do indicate that a difference between the two acclimation groups can be observed at lower temperatures of measurement as well as at 22°.

Discussion

Previous studies on the effect of varying environmental temperatures in toadfish have shown: (1) that fish acclimated for one–two weeks at 10° show about 75% greater protein synthetic capacity in liver than control 21° fish when incorporation of radioactive amino acids is measured *in vivo* at 21° (Haschemeyer, 1968); (2) that an aminoacyl transferase functioning in the protein synthetic pathway is increased in cold-acclimated fish (Haschemeyer, 1969a); and (3) that polypeptide chain assembly is significantly faster in cold-acclimated animals (Haschemeyer, 1969b). These changes are not observed in toadfish subjected to only one or two days at 10°. In every case the changes appear to be associated with a compensatory response to the lower temperature, *i.e.,* temperature acclimation.

The present studies were carried out to determine whether toadfish, when assayed for oxygen consumption, would show physiological compensation of metabolism comparable to that observed in other vertebrate fish after cold acclimation and consistent with the molecular changes occurring in the protein synthetic system. As in the previous investigations, acclimation temperatures of 10° and 21° were used. These are within the expected range for capacity adaptation (with maintenance of normal functions), as discussed by Prosser (1967) and Roberts (1967). The results show that at a measurement temperature of 22°, cold-acclimated (10°) toadfish have a significantly greater rate of oxygen consumption (about 42%) than control (21°-acclimated) fish. It therefore appears that toadfish develop at least partial metabolic compensation after 9–12 days at 10°; whether a longer period might produce a more complete compensation, as in sunfish (Roberts, 1967), is not known. The magnitude of the effect falls within the range observed for other poikilothermic vertebrates (Prosser, 1962) and is comparable to the changes observed at the molecular level in the toadfish. Further studies on the mechanism of these changes are in progress.

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Summary

Oxygen consumption of toadfish was measured in a closed respiratory chamber using a polarographic oxygen electrode. Cold-acclimated (10°) toadfish were found to consume oxygen at a rate 42% greater than control 21°-acclimated fish, when both groups were measured at 22°. The results indicate that toadfish are capable of partial metabolic compensation in response to low temperatures, comparable to that observed in other poikilothermic vertebrates. The overall metabolic effect appears to correlate well with changes observed in the protein synthetic system of toadfish liver under the same acclimation conditions.

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THE RESPONSE OF *PARAMECIUM BURSARIA* TO POTENTIAL ENDOCELLULAR SYMBIONTS[1, 2]

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The series of events leading to experimental endocellular symbioses must begin with ingestion and result in cellular interaction between host and symbiont. In the initial step the feeding mechanism, feeding specificity, and chemoreceptors probably all play some role in screening potential symbionts.

The ability of *Paramecium* and other protozoans to feed selectively has been pointed out by Bragg (1936), Mast (1947), and Nelson (1933).

In the paramecium-alga complex of *Paramecium bursaria* the interacting populations can be separated, recombined and re-combined to give novel combinations (Oehler, 1922; Siegel and Karakashian, 1959; Siegel, 1960; Karakashian, 1963). From these works it can be seen that *P. bursaria* does not exhibit an all-or-none response to foreign algae, but does demonstrate some degree of specificity which is manifest in the size of the intracellular population that develops following "infections."

Also it has been demonstrated that each member of the complex can exist independently of the other (Karakashian, 1963) and therefore each may be considered a facultative symbiont. In addition, the symbiotic complex can be cultured in the absence of an exogenous food supply (Loefer, 1936).

This study was undertaken: (1) to determine the role of the feeding response in establishing an endocellular symbiosis; (2) to test the response of *P. bursaria* to algae of several genera of the chlorococcales representing a spectrum of nutritional types.

Materials and Methods

Stock cultures

The cultures used were obtained from the following sources: *Paramecium bursaria* 32w, the alga isolated from *P. bursaria* 32g (Siegel, 1960), and the food organism *Aerobacter cloacae* were provided generously by Dr. R. W. Siegel and Dr. S. J. Karakashian; *Chlorella vulgaris* 263, *Chlorella variegata* 256, *Trebouxia erici* 912, *Chlorococcum minutum* 117, and *Prototheca zopfii* 328 were obtained from the Indiana Culture Collection (Starr, 1960); the *Chlorella vulgaris* Emerson strain was obtained from Dr. David Appleman.

Culture methods (paramecia)

In general the methods used for the culture of *Paramecium bursaria* were the same as those described by Sonneborn (1950) for the culture of *P. aurelia*.

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[1] Part of a thesis submitted to the Graduate Faculty of Rutgers The State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] The author wishes to acknowledge the advice, assistance and encouragement of Dr. Edwin T. Moul during the course of this study.

The bacterized culture medium was a lettuce infusion inoculated with the washings from a three- or four-day-old bacterial slant and incubated for 24 hours at 25° C before use. All cultures of paramecia were maintained at 25° C on a 12-hour diurnal cycle with a light intensity of 200 foot candles.

Culture methods (algae)

All cultures of algae were maintained on an organic medium described by Loefer (1936) as suitable for the axenic culture of *P. bursaria.*

Experimental methods

For experiments involving the infection of algae into paramecia, the paramecia were taken from a mass culture, centrifuged, and added to a mixture containing 1 ml of freshly bacterized lettuce infusion and 4 ml of the appropriate alga, washed from a 10-day-old agar slant. This procedure yielded cell concentrations as follows: *ca.* 50 paramecia per cc, *ca.* 10^9 bacteria per cc, and from 10^7 to 10^9 algae per cc. These preparations then were allowed to stand under standard culture conditions for the period indicated, at which time a single green paramecium was isolated, washed 4 times in lettuce medium, then removed and allowed to form a mass culture. These isolations were done in triplicate.

Paramecia were counted in a Sedgwick-Rafter counting chamber; algae were counted in a modified Neubauer chamber; bacteria were counted by serial dilutions and emulsion agar plating.

For experiments involving ingestion, 10-ml aliquots were taken from a mass culture of paramecia, and centrifuged for 15 minutes at 2,000 rpm. Starch grains or carmine to be ingested were suspended in bacterized lettuce infusion. In cases where algae were to be ingested, these too were centrifuged and resuspended in bacterized lettuce infusion. The non-living particles or algae or both in bacterized medium were then added to the centrifuged paramecia. After the appropriate length of time, samples were withdrawn and placed within a ring of 5% methylcellulose, cps 15, on a glass slide, and observed under 100 × magnification. Unless otherwise noted 45 animals were observed. Animals were considered positive if they contained even a single particle.

RESULTS

Ingestion

Experiments designed to measure the ingestion of *Chlorella* 32g yielded irregular results. In general great variability was observed in the ability of the paramecia to ingest this alga. Thus it was necessary to determine if the variability was a characteristic pattern in the feeding response of the paramecia or if this variability represented a type of selection on the part of the paramecia.

According to the methods described suspensions of carmine were prepared so as to give concentrations of particles comparable to the concentration of algal cells used, from 10^7 to 10^9 per cc. The paramecia consistently ingested particles of carmine. When placed in these suspensions, they immediately formed many carmine-containing vacuoles. After remaining in these suspensions for 24 hours they continued to pack themselves with these particles, and presented a striking picture with as many as 8 or 10 bright red vacuoles.

The possibility exists that the variability of ingestion observed in the presence of algae might be explained on the basis of an algal metabolite that inhibits ingestion. To test this possibility suspensions of carmine were prepared in supernatants of 20-day-old algal cultures of *Chlorella* 32g. It was found that the supernatant in no way altered the ingestion of carmine. The paramecia still continue to form many carmine-containing vacuoles.

The particles of carmine used, although not carefully measured, were small enough to exhibit Brownian movement. In contrast to the small size of the carmine particles, the chlorella range in size from 4 to 6 μ. To test the possibility that the carmine particles are being swept passively into the food vacuoles and that the algae are too large to be ingested readily, it was necessary to measure the ingestion of larger particles.

TABLE I

Nutritional types of the algae used

Alga	Nutritional type	Reference
Chlorella sp. (from *P. bursaria*)	facultative heterotroph	Loefer, 1936
Chlorella vulgaris	facultative heterotroph	Algeus, 1948; Van Niel, 1941
Chlorella vulgaris Emerson	obligate autotroph	Finkle *et al.*, 1950; Killam and Myers, 1956; Griffiths, 1961
Chlorella variegata	facultative heterotroph	Fritsch, 1935
Trebouxia erici	facultative heterotroph	Ahmadjian, 1960
Chlorococcum minutum	obligate autotroph	Parker *et al.*, 1961
Prototheca zopfii	obligate heterotroph	Barker, 1935, 1936

Suspensions of Argo corn starch were prepared, and the paramecia mixed with these suspensions. It was observed that the paramecia readily ingested starch grains of all sizes, including particles as large as 14–15 μ.

By a process of crude differential sedimentation, particles of fairly uniform size were obtained by suspending 1 gram of corn starch in a graduate cylinder containing 100 ml of fluid, allowing this to stand for 10 minutes, and then removing 1-ml aliquots from the top layer of fluid. In this manner particles of starch ranging in size from 12–15 μ were obtained. Paramecia were placed in these suspensions, and allowed to feed. Samples were removed and the paramecia were killed with iodine. The paramecia consistently ingested these large particles.

Ingestion of algae

The algae used in these experiments represent a series of chlorococcalean algae of diverse nutritional requirements. These algae are listed in Table I, along with their nutritional type and references to papers where the nutritional requirements of these algae are reported.

Table II summarizes the experiments in which ingestion of these various algae was studied. It can be seen that all of the algae tested, except *Prototheca zopfii,* are ingested to some extent. These data also suggest that the factors involved in

TABLE II

Ingestion of algae by Paramecium bursaria 32w

Particle	Per cent of paramecia ingesting particle
Starch	100
Chlorella 32g	93
C. vulgaris	53
C. vulgaris Emerson	53
C. variegata	47
Trebouxia erici	14
Chlorococcum minutum	27
Prototheca zopfii	0

the ingestion of algae are not the same factors involved in the ingestion of non-living particles. The ingestion of non-living particles is characterized by uniformity, while the ingestion of living particles varies. From observations, it appears that this variation is independent of cell concentration and cell size.

Selection

The above experiments demonstrate the consistency with which paramecia ingest non-living particles. This stands in dramatic contrast to the variation observed when the paramecia are fed algal cells. To further determine the role of the feeding response in the ingestion of algae, experiments were designed to measure the ability of the paramecia to select between different particles when present in approximately equal concentrations.

Paramecia were placed in suspensions of carmine or *Chlorella* 32g or a mixture of carmine and *Chlorella* 32g. These experiments demonstrated that when the algae were present alone, or in a mixture with carmine, they were ingested to a limited extent, while the carmine when present alone or in a mixture was ingested by the entire population of paramecia. A similar experiment was carried out using

TABLE III

Selection between various particles by Paramecium bursaria 32w

Experiment	Per cent of paramecia ingesting particle	Per cent of paramecia ingesting algae
Carmine	100	—
Chlorella 32g	—	46.5
Carmine + *Chlorella* 32g	100	40
Experiment		
Starch	100	—
Chlorella 32g	—	20
Starch + *Chlorella* 32g	100	20
Experiment		
Starch	100	—
Prototheca zopfii	—	0
Starch + *Prototheca zopfii*	100	0

starch grains that approximate algal cells in size, or *Chlorella* 32g or a mixture of the two. The results are similar in that the ingestion of either the starch grains or the algae is independent of the presence of the other particle. This was further confirmed by feeding the paramecia a particle that never was ingested, the colorless alga *Prototheca zopfii.* As in the other experiments this alga was fed alone and in a mixture with a particle that always was ingested, starch grains. In short, the presence of another particle does not alter the ingestion pattern for either particle separately. The results are summarized in Table III.

Clearly these data indicate the ability of the paramecia to ingest a variety of particles, and they reflect the selective ability of *Paramecium bursaria.*

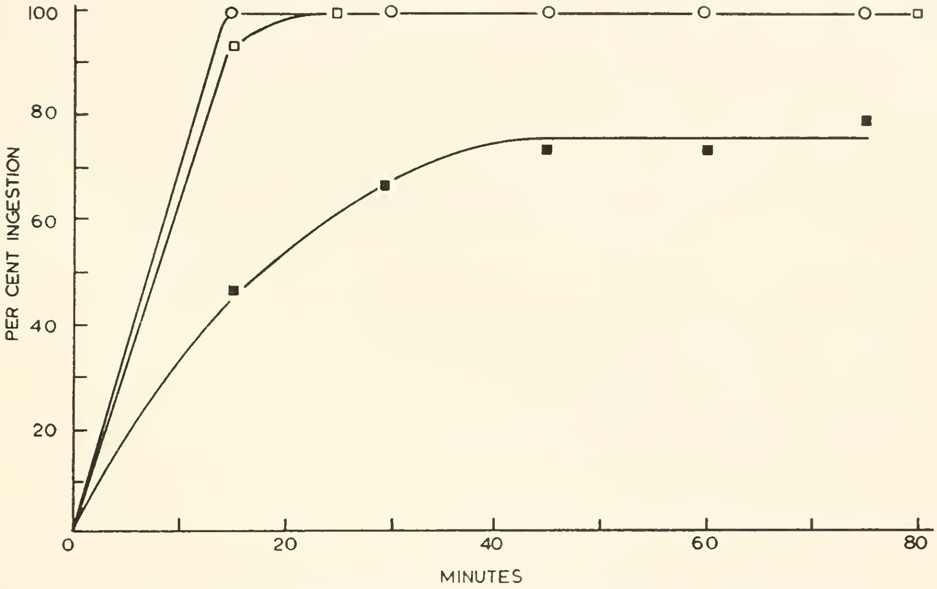

FIGURE 1. The ingestion of light-grown and dark-grown *Chlorella* 32g. Open circles are controls, ingestion of starch grains in the light or in the dark. Open squares of light-grown *Chlorella* 32g. Closed squares are ingestion of dark-grown *Chlorella* 32g. Ordinate is the per cent of paramecia ingesting each particle. Abscissa is time in minutes.

Light and dark experiments

In trying to determine what factors are involved in the ingestion of algae, several chance observations suggested that the paramecia actively ingest the algae at the beginning of a light period.

Based on these observations, the following experiments were undertaken. The algae used were harvested from a twenty-day-old culture of *Chlorella* 32g. Light grown algae were harvested after 105 minutes in the light following a 12-hour dark period. Dark-grown algae were maintained for 15 hours and 45 minutes in the dark prior to use. Dark-grown algae were centrifuged, re-suspended, mixed with paramecia, sampled and killed on a slide, all in the dark to eliminate the possibility that the paramecia might feed while on the slide when being observed in the light. The paramecia were killed with Patterson's fixing fluid (Zuck, 1959). The dark and light samples were run simultaneously. As controls for these experiments ingestion of starch was measured in the light and in the dark. These paramecia were killed with iodine. In these experiments 30 animals were observed.

These results are summarized in Figure 1. There is no difference in the ingestion of starch in the light or in the dark, and by the time of the first measurement all of the paramecia had ingested starch, and continued to ingest starch for the duration of the experiment. The rate of ingestion of light-grown algae almost equalled the rate of ingestion of starch; the two curves show almost the same steep slope. In conrast, the dark-grown algae at the time of the first measurement were ingested almost 50% less than light-grown algae. At the point where these curves level out, there is approximately a 25% difference between light-grown algae, and the controls.

These data suggest that the paramecia feed selectively and that the feeding mechanism is sensitive to some physiological condition of the alga.

TABLE IV

Percentage of alga-containing progeny descendant from a single infected individual after 13 weeks

Infecting alga[1]	Per cent of progeny that contain the alga
Chlorella 32g	100
Chlorella vulgaris 263	100
Chlorella vulgaris Emerson	61
Chlorella variegata 256	40
Trebouxia erici 912	—
Chlorococcum minutum 117	31
Prototheca zopfii 328	—

Fate of the algae within the paramecia

All of the algae tested except *Prototheca zopfii* are ingested (Table II). However, the fate of the algae varies; some are egested later, apparently undamaged, others are digested visibly, and still others seem to multiply within the paramecia.

Artificial infections and persistence of algae within paramecia

Paramecia containing algae were isolated from mixtures of paramecia and algae that had been allowed to stand for 18 days under standard conditions. No isolations were made in the case of *Trebouxia erici* or *Prototheca zopfii*. These paramecia were isolated, washed four times according to the method described by Sonneborn (1950) and allowed to form mass cultures. The persistence of algae in later generations was studied. The results are listed in Table IV. Although all cultures were started from paramecia that contained algae, only in the case of *Chlorella* 32g and *Chlorella vulgaris* 263 has distribution of algae to all of the paramecia been achieved.

All of the cultures were fed at the same interval to allow for equal growth rates of the paramecia. Therefore, the unequal distribution of algae to the progeny of paramecia infected with *C. vulgaris* Emerson, *C. variegata,* and *Chlorococcum minutum* might be explained by (1) egestion, (2) digestion, or (3) simply outreproducing the algae. Probably the growth of *Chlorella* 32g and *C. vulgaris* 263 are synchronized with the growth of the paramecia and thus the algae are able to maintain very high intracellular populations.

Discussion

Recent histochemical studies on the food vacuoles of paramecia indicate that functional vacuoles containing non-nutritive particles are formed (Mueller *et al.,* 1965). However, there is no literature specifically relating to the food vacuoles of *P. bursaria.* There is evidence that the feeding responses of many protozoa involve surface reactions: *Balantidium coli* are selective as to quality (Nelson, 1933); *Podophyra collini* demonstrates a preference for ciliates based on an enzyme catalyzed reaction involving the tentacle of the suctorian and the ciliate (Hull, 1961); electron micrographs reveal an initial attachment phase of the particles to the membrane surface during the process of phagocytosis (Brandt and Pappas, 1960). These facts might account for the selective differences in the feeding response of *P. bursaria* to light-grown and dark-grown *Chlorella* 32g, as well as for the failure of paramecia to ingest *Prototheca zopfii.*

Artificial infections

It appears that the relationship between *P. bursaria* and its intracellular algae is neither intimate nor permanent (Table IV), but the infection of *P. bursaria* may depend on the physiological state of the potential symbiont. This is somewhat similar to the infection of *P. aurelia* with kappa particles in that certain stages of the "life cycle" of kappa may be more infective than others (Sonneborn, 1959; Tallan, 1959).

Adaptations of the algae

From the results of this study it appears that various algae, at least under some conditions, are ingested, resist digestion and are capable of growth in the cytoplasm, while only the naturally occurring chlorella and *Chlorella vulgaris* 263 (of those tested) are able to achieve synchronous growth. Perhaps the rapid rate of growth of chlorella serves as a synchronizing mechanism between the paramecium and the algae thereby predisposing the algae to its "parameciumized" fate.

The nature of the interaction

Associations clearly recognizable on a morphological basis as symbioses are widespread and provide not only novel and unique combinations in nature, but are interesting from an evolutionary point of view. Quispel (1951) and Lederberg (1952) have used the term *symbiosis* to describe interactions below the organism level of organization, for example, the interactions between cellular organelles and the cytoplasm.

There is a continuum of interactions that grade between mutualistic symbiosis and parasitism, and the more these relationships are studied, the more it becomes apparent that these interactions are by no means stable and the balances that exist between host and symbiont can be upset easily (Dubos and Kessler, 1963). To illustrate this, lichens have often been regarded as an example of commensalism but it has been shown that cells of the phycobiont are penetrated by haustoria of the fungus (Moore and McAlear, 1960; Ahmadjian, 1962). Thus it should not be assumed that organisms enter into symbiotic associations for the mutual benefit of each other (Caullery, 1952; Droop, 1963).

Recently, Karakashian (1963) has demonstrated in *Paramecium bursaria* that under certain conditions the algae increase the growth rate of the paramecia. This increase is probably mediated by some photosynthetic product. In addition, if one assumes that under some conditions the development of an intracellular population of algae is also enhanced as a result of the protection afforded the algae in this microhabitat, then, according to the classification of interactions of Burkholder (1952), this relationship might be termed protocooperation. However, these events do not prevail under all conditions. Under stress conditions for either host or symbiont (food limiting, or darkness) the intracellular population will decrease in size (Karakashian, 1963). Under some conditions the algae are egested (Oehler, 1922), and at other times they appear to be digested.

If the alga contributes a photosynthetic product to the complex, then clearly in the dark the interaction will be different from what it is in the light. If in turn the paramecium contributes a carbohydrate to the alga, then here too the interaction will be different in the light and in the dark, since none of the algae, except the obligate heterotroph is dependent on a preformed carbohydrate. Clearly the balances in this relationship are sensitive, and are upset easily. Factors involved must include the physiological condition of the alga, the availability of food for the paramecium, and the intensity of light. Varying any of these conditions alters the interaction between the host and its "plasmid" (Lederberg, 1952).

Karakashian (1963) considers the exchange of metabolites and the alteration of the growth pattern of the symbiotic complex as evidence that the host and its plasmid are a well integrated functioning unit in nature. Muscatine and Lenhoff (1963) have shown that 10% of the carbon fixed by the *Chlorella* of *Chlorohydra viridissima* appears in the host animal. Can the mere exchange of metabolites be taken as evidence for integration between a host and its symbiont? Zabka and Lazo (1962) have shown the reciprocal exchange of radio-phosphorus between a myxomycete, *Fuligo cinera* and an alga, *Chlorella xanthella,* two organisms that do not exist as a symbiotic complex.

In pure cultures of various Chlorophyceae, appreciable amounts of the total carbon fixed appear in soluble form in the external medium. In *Anabaena cylindrica* up to 1.4% of its dry weight is liberated as extracellular pentose (Fogg, 1952). With these works in mind it is not at all surprising to find an exchange of metabolites occurring between the paramecium and the algae, in the *P. bursaria* complex.

It is also necessary to consider whether organisms living together always interact. It is not likely that the encysted flagellates and *P. trichium,* reported by Wenrich (1926), occurring together, interact with each other.

It appears that the relationship between *P. bursaria* and its endocellular symbiont, *Chlorella* sp., typically is ephemeral. The establishment of this relationship depends on ingestion. The fate of the algae within the paramecium varies. Exchange of metabolites clearly must occur under some conditions, but the actual interactions vary, and can not easily be classified in any conventional system. The interactions are dynamic, and the equilibria are upset easily.

SUMMARY

1. *Paramecium bursaria* ingests a variety of particles, but this ingestion is not random. The paramecia select between different particles of approximately the same size when present in approximately equal concentrations.

2. The paramecia ingested six of the seven species of algae tested. Five of these six are maintained to some extent in the cytoplasm of *P. bursaria*. Maintenance of the alga in the cytoplasm seems independent of the nutritional type of the alga.

3. Of all the algae tested, only the naturally-occurring species of *Chlorella* and a free-living strain of *Chlorella vulgaris* have been established as symbionts, the criterion being the distribution of algae to all of the progeny of the paramecium. The adaptations of the algae to this niche are discussed.

4. The role of (a) selective feeding by the paramecia, and (b) the physiological condition of the alga in establishing this relationship are discussed.

5. The nature of the interaction between *Paramecium bursaria* and its endocellular symbiont is discussed. Apparently this relationship is unusual since it lacks specificity and permanence, two traits characteristic of most symbioses.

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RESPONSES TO SALINITY CHANGE AS A TIDAL TRANSPORT MECHANISM OF PINK SHRIMP, *PENAEUS DUORARUM*[1]

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Many species of penaeid shrimp carry out extensive movements during the course of a life cycle. Larval and post-larval stages may travel from offshore spawning sites to inshore nursery areas, frequently deep into estuaries or mangrove swamps where they remain until they return to offshore waters as juveniles or subadults. This investigation is concerned with the method whereby the postlarvae and juveniles of *Penaeus duorarum* Burkenroad carry out their respective movements into and out of nursery areas.

Off southern Florida spawning in this species occurs in the vicinity of the Tortugas shrimp fishery grounds approximately 60 to 100 miles S.E. of the Everglades (Munro *et al.* MS). The early postlarval stages (total length 0.8 to 1.4 cm) arrive in the estuary where they remain until they return to deeper waters as juveniles or subadults (t. l. approximately 7.0 to 10.0 cm). Sampling data (Tabb *et al.*, 1962; Hughes MS) show clearly that the arriving postlarvae are predominantly collected from night flood tides while the juveniles are taken on the night ebb tides. This observation agrees with that of St. Amant *et al.* (1966) for the brown shrimp, *Penaeus aztecus* Ives, the postlarvae of which move into Louisiana estuaries principally on flood tides.

By selective use of tidal currents as transporting media the movements of animals into and out of inshore waters are facilitated and the position of certain species within such areas maintained (reviews, Verwey, 1958, 1960; Stieve, 1961). In the few experimental investigations of these movements it has been found that some physical or chemical change associated with the change in tide elicits a corresponding behavioral change, enabling the animal to utilize one or other tide for its displacement while avoiding displacement by the alternate tide (Creutzberg, 1961; Haskins, 1964).

In the inshore waters usually occupied by penaeid shrimp the factor which changes most with change of tide is salinity. In the canal from which the material for this study was collected, salinity changes between tides were seldom less than 5‰ and in summer were frequently greater than 10‰. On the supposition, therefore, that responses to salinity changes may contribute to the tidal transport mechanism, they were investigated in both postlarvae and juveniles.

METHODS AND APPARATUS

All shrimp were colleced at night from Buttonwood Canal in the estuary. Discovery-type plankton nets were suspended from a bridge into the strong tidal currents. Depending on the season and the state of the tide, it was possible to

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[1] Contribution No. 977 from the Institute of Marine Sciences, University of Miami.

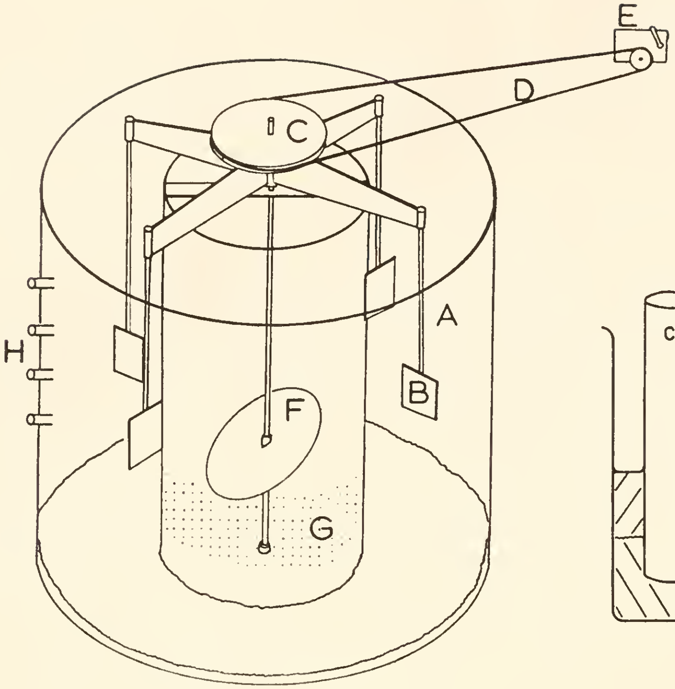

FIGURE 1.

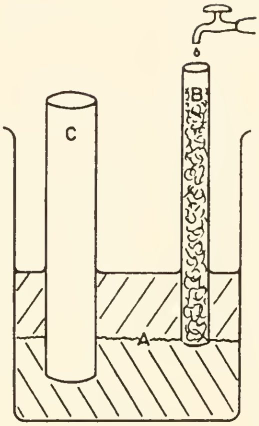

FIGURE 2.

FIGURE 1. Current chamber. For details see text.

FIGURE 2. Apparatus to investigate perception of salinity differences by postlarvae. For details see text.

collect several hundred postlarvae or upwards of five juveniles in each net during a ten-minute haul. The shrimp were placed in five-gallon jars and taken immediately to the laboratory. Two types of apparatus were used.

1. A current chamber (Fig. 1), a modification of that used by Creutzberg in studying eels (1961). Two "Plexiglas" cylinders were set, one within the other to form a circular canal between them (A). A current was created within the canal by means of four paddles (B) attached to a central pulley (C) connected by a rubber drive belt (D) to a variable speed motor (E). Reductions in the salinity of the water of the canal were effected by running distilled water into the inner cylinder where it was mixed by an angled disc (F) attached to the central pulley and allowed to penetrate the canal through numerous small holes (G). Salinity was monitored at regular intervals from water siphoned from a level within the chamber approximately where the shrimp were swimming. The bottom of the canal was covered with 5 cm of beach sand, and the water level was altered and maintained by a series of outflow points (H). The entire apparatus was housed in a light-tight enclosure and illuminated by day by a 150 w flood lamp, the beam from which was diffused by deflection off the white roof of the enclosure. A 10 w red bulb, casting only enough light to permit observation at night, was suspended over the center of the apparatus and kept on constantly.

2. The perception of salinity differences was investigated in a simple apparatus (Fig. 2) in which a discontinuity barrier (A) could be created between bodies of water of differing salinity. Water was run to a depth of 3 cm in a 500-ml beaker. Onto the surface of this, water of lower salinity was slowly run through a tube filled with cottonwool and sand (B), which prevented turbulence. Effective barriers were thus formed between waters differing in salinity by only 1‰. Postlarval shrimp were introduced through a second tube (C) directly into the water of higher salinity, and their reactions to the discontinuity barrier were observed and recorded. Dyeing techniques in one or other bodies of water showed that the barrier was not rapidly broken down, even when frequently penetrated by swimming postlarvae.

Results

Experiments in the current chamber were generally conducted on newly caught specimens, although occasionally material which had been in the laboratory for a week or more was used. Seven (Series I) or eight (Series II) shrimp were placed

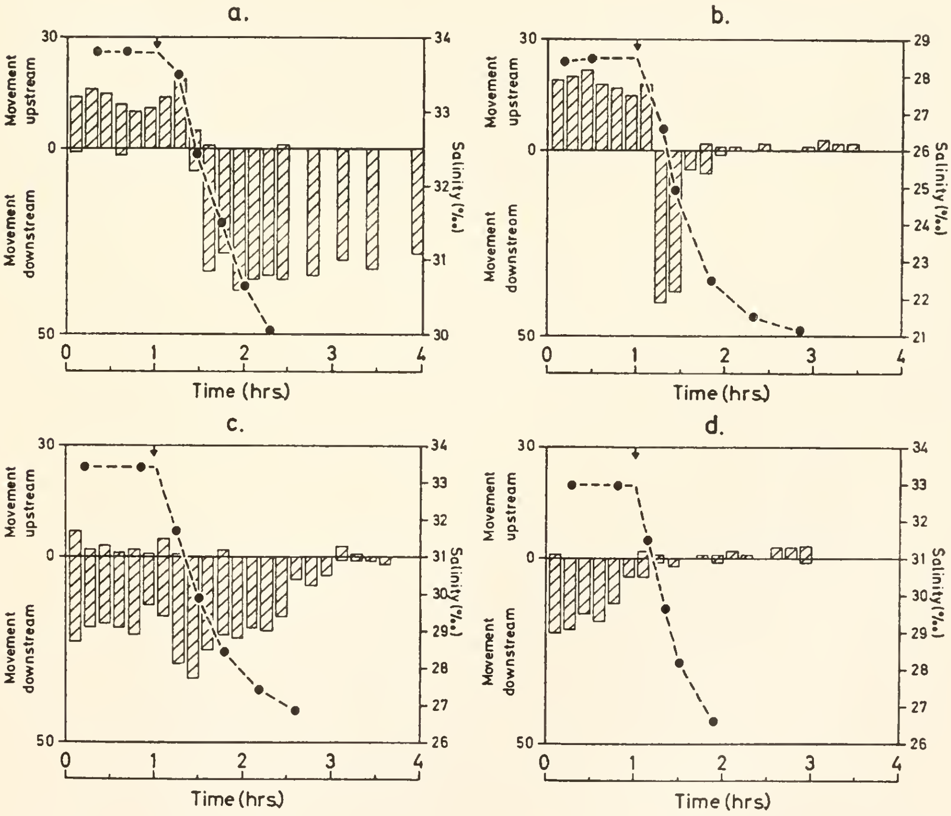

Figure 3. The effect of a decrease in salinity on the swimming of juveniles. Salinity is indicated by the broken line and the time of onset of the decrease by the arrow. For further explanation see text.

in the apparatus at one time. The salinity of the water was between 28‰ and 34‰. The current was maintained at 12 cm/sec. This was arbitrarily chosen as a speed against which the shrimp could swin but which did not erode the substrate. The direction and extent of swimming of the shrimp were recorded at intervals of 10 or 20 minutes for several hours before imposing a salinity decrease, and for up to 4 hours following the decrease.

Responses of juveniles

Juvenile shrimp maintained in currents of water usually orientate and actively swim upstream. However, when they are deprived of food for a few days or when they are in polluted water, downstream swimming occurs. In addition, downstream swimming sometimes takes place in the apparent absence of any environmental change. These apparently spontaneous reversals in sign of rheotaxis are the subject of another paper (Hughes, in preparation).

In these experiments there was usually a conspicuous change in behavior following a decrease in salinity within the current chamber. If swimming against the current the shrimp turned and swam downstream (Fig. 3a, b), or if they were already swimming downstream they increased the speed of downstream swimming (Fig. 3c). There were a few exceptions to this (Fig. 3d), apparently when the salinity decrease was imposed towards the end of the downstream swimming phase. This active swimming sometimes persisted for several hours, or for less than an hour. The more rapidly the salinity was decreased the more marked was the response of the shrimp. A slow reduction in salinity (2‰ in 40 min) failed to elicit the response produced by the same reduction taking place in 20 min. A

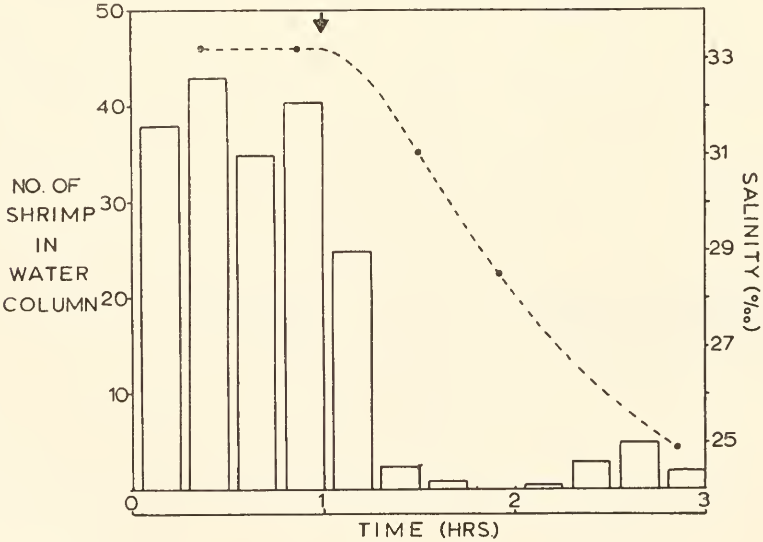

FIGURE 4. The effect of a decrease in salinity on the occurrence of postlarvae within the water column. Salinity is indicated by the broken line and the time of onset of the decrease by the arrow.

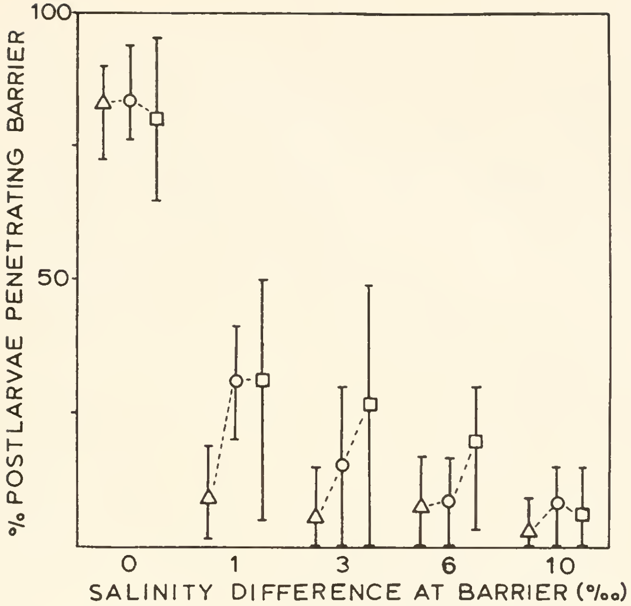

FIGURE 5. The responses of postlarvae at salinity discontinuity barriers of various magnitude. The figure indicates the extent of penetration of the barrier during the first (△), second (○) and third (□) five-minute period following exposure to it. In each case the mean of three experiments is given; the vertical lines indicate the range.

greater (10–15‰) and more rapid decrease was required to elicit this behavior in shrimp which (for reasons stated above) had swum downstream earlier in the night or which had been held in the laboratory for a few weeks. In these cases, downstream swimming, once elicited, was usually of short duration (less than 30 min).

The consistency and predictability of the variation in response suggests a "changing responsiveness" to the stimulus of salinity decrease. This was not critically examined and remains supposition.

Responses of postlarvae

Similar salinity changes were imposed on postlarvae. The current speed was maintained at 5 cm/sec, a velocity against which the smaller animals could swim.

A salinity decrease of 2 to 3‰ caused them to drop out of the water column and confine their activity to the substrate and the water just above it (Fig. 4).

In an earlier report (Hughes, MS) it was stated that a decrease in salinity caused a decrease in activity of postlarval pink shrimp. Although in subsequent experiments this was sometimes the case, it was by no means invariable: frequently the shrimp remained active on and just above the substrate.

Results were not always consistent; at times a greater decrease was required before the postlarvae would drop out of the water column. Again it is possible that a rhythm controlling the responsiveness to salinity decrease is present. This merits future investigation.

The perception of salinity differences by postlarvae

From previous experiments it was apparent that both juvenile and postlarval shrimp would perceive and respond to changes in salinity as small as 2‰ or 3‰. In the case of postlarvae this was verified and further defined by experiments conducted in the apparatus in which a discontinuity barrier was created between bodies of water differing in salinity. Prior to the experiments the postlarvae were kept in water of 33‰ for 24 hours. Ten individuals were then placed into water, also of 33‰, in the apparatus (Fig. 2), and their responses were recorded at the barrier between this water and bodies of water of 23‰, 27‰, 30‰, and 32‰. Normally postlarvae placed in a beaker swim up and down within the water column, but in this apparatus after penetrating the barrier they would usually sink motionless to the bottom before again swimming upwards.

Control experiments, in which no barrier was present, were also conducted. The results (Fig. 5) clearly indicate that the barrier is perceived even when the salinity difference is as low as 1‰. Usually after repeated contacts the shrimp penetrated the barrier and ultimately swam on either side of it. The increased incidence of penetration during each successive five-minute period (Fig. 5) is a result of these repeated contacts. Lance (1962) in her study of the responses of a number of zooplankters to salinity discontinuity barriers, recorded similar sinking to the substrate following penetration of low salinity water. She also found that

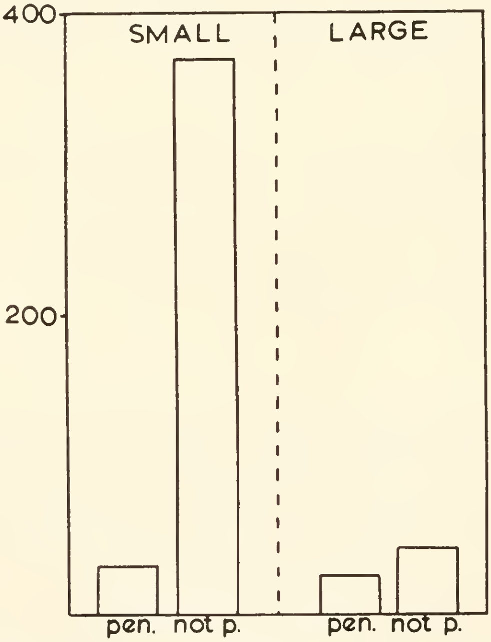

Figure 6. A comparison of the responses of "small" and "large" postlarvae at a discontinuity barrier (4‰ difference). The bars represent the total postlarvae which, during three separate fifteen minute trials penetrated (pen.) or, having swum up to the barrier, did not penetrate (not p.) it.

after a period of exposure to any particular salinity the number of animals swimming in the upper half of each diluted column progressively increased during the course of the experiment.

The dependence on postlarval size

In the foregoing experiments it appeared that the behavior of the postlarvae at the discontinuity barrier was dependent on their size to some extent. This supposition was tested by comparing the responses of two size-groups, each comprised of 12 individuals, at a barrier between waters of 34‰ and 30‰. The experiment was repeated three times using fresh groups of animals.

The postlarvae were selected arbitrarily as "small" or "large." In the first experiment each animal was measured and rostral spine counts taken. The "small" postlarvae ranged in total length from 9.2 to 11.1 mm (mean 9.8) with a mean rostral count of 6. The "large" ranged between 11.5 and 13.1 mm (mean 12.2) with a mean spine count of 8. Records of growth in the laboratory indicated that these size differences represented approximately a week's growth. In the following two experiments the criteria for the sizes were the same as in the first experiment.

The results (Fig. 6) are expressed as the total number of postlarvae which, on reaching the barrier, penetrated or failed to penetrate it during a 15-minute period. These show clearly that smaller postlarvae are less likely to penetrate the barrier than their larger counterparts, and that they are, in addition, more active.

Discussion

Investigations of the responses of both postlarval and juvenile shrimp to changes in salinity suggested a mechanism whereby the tide-associated movements may be carried out by this species.

Juveniles

Juvenile shrimp habitually orientate into and actively swim against a current but it was found that a relatively small salinity decrease (2 to 3‰) was sufficient to reverse the sign of the rheotaxis, causing them to turn about and swim downstream. The downstream swimming often gave way to passive drifting with the current which could continue for four to five hours in the experimental situation.

Upstream and downstream swimming differs in a manner which is certainly highly adaptive. Upstream swimming occurred as a series of short "hops" close to the substrate with constant maintenance of orientation by returning to the substrate, whereas downstream swimming occurred at varying depths in the water column and without constant reference to the substrate. In the experimental situation the proximity of the walls, the substrate, and the movement of the paddles could provide reference points outside the current enabling downstream orientation. But in nature, with greater depths, current speed and turbulence, the shrimp, having lost contact with the substrate would not be able to orientate at night, but would be passively displaced by the prevailing ebb tide. Even in the current chamber active downstream swimming gave way to passive drifting sometimes for hours. In all probability the juvenile shrimp taken in ebb tide samples are not orientated downstream but are being passively displaced, perhaps following an initial period of downstream swimming. (The marked downstream orientation which initially followed a salinity decrease indicated a clear reversal in the sign of the rheotaxis, and not merely a suppression of the positive orientation to current.)

The occurrence and duration of downstream swimming depended on the magnitude and rate of salinity decrease, the time the shrimp had been maintained in the laboratory, and their swimming behavior prior to the decrease. However, the consistency of the response and the predictability of its modifications suggested a periodically changing responsiveness to the salinity decrease. And on the basis of the many occasions in which downstream swimming persisted long after the changes had ceased, it seems possible that the decrease was only the "trigger" releasing downstream swimming. These points were not critically examined and remain supposition. It is evident, however, that in an estuarine environment, where frequent fluctuations in salinity occur, there would be adaptive advantage in temporarily synchronizing downstream swimming with the time of the ebb tide.

Reversals of rheotactic response have been little investigated, but precedents do occur in the literature. Creutzberg (1961) working on the elver, *Anguilla anguilla* L., found a reversal similar to that described here. He showed that elvers would swim against a current containing (p. 336) "an attractive substance, . . . which is presumably an odor specific to inland water," but that when this "odor" was decreased they would swim with the current. Thus in nature they would swim inshore with the flood tide, but avoid displacement by swimming against the ebb. Keenleyside and Hoar (1954) showed that increases in temperature caused reversals of rheotaxis in three species of juvenile salmon, and a temperature increase of 5° C was shown by Beauchamp (1937) to induce a positive rheotaxis in the turbellarian *Planaria alpina.*

Postlarvae

Experiments which tested postlarval responses at discontinuity barriers between waters of different salinities showed clearly an ability to perceive and respond to a difference of as little as 1‰. In water of higher salinity (such as normal seawater) the postlarvae are active in the water column. If, however, the water becomes stratified with water of lower salinity run onto its surface, the shrimp are confined nearer the substrate through an apparent "reluctance" to penetrate the less saline water. This dropping to the substrate was therefore not, as had been previously suggested (Hughes, MS), merely a reduction in activity similar to that reported by Lance (1962) for zooplankters moving into regions of diluted sea water, but indicated an "aversion" to penetrating the water of lower salinity. Clearly these reactions in nature would limit displacement to the time of the flood tide. In normal sea water the postlarval shrimp are active in the water column and, being incapable of withstanding even slow currents they would be displaced by the prevailing tide. With decrease in salinity during the ebb tide the postlarvae are excluded from the water column and remain on or near the substrate where they are better able to maintain position and are less readily displaced.

The marked "aversion" for waters of lower salinity and the high level of swimming activity exhibited by "small" postlarvae become considerably reduced within a period of approximately one week. It is probable that these changes occur at a time when the original response is no longer necessary. Sampling data (Tabb *et al.*, 1962) from two points three miles apart along a canal leading into the Everglades indicate that postlarvae may be transported this distance in only one flood tide, and that the larvae may therefore penetrate deeply into estuaries within only a few days of their arrival in inshore waters.

A similar effect of salinity change on activity probably maintains oyster larvae within inshore waters (Haskins, 1964), and Lance (1962) showed that a number of zooplankters were restricted to certain ranges of salinity in stratified estuaries by their responses to discontinuity barriers, suggesting (p. 131) that "discontinuity layers will influence the dispersal of zooplankton by modifying the position of individuals relative to prevailing water currents." Further, Grindley (1964) confirms that low salinity surface waters in an estuary (River Test, England) prevented most species of estuarine zooplankton from upward movement. He also showed experimentally that under the same conditions the copepod *Pseudodiaptomus hessei* (Mrázek) was prevented from carrying out its normal vertical migration and confined instead to deeper layers.

The tidal transport mechanism—Summary

The method whereby responses to salinity changes occurring with changes in tide may facilitate the respective displacements of postlarval shrimp into and out of inshore nursery areas is summarized as follows. In the case of juvenile shrimp a positive rheotaxis is present throughout flood tides when "normal" sea water salinities prevail, but when salinity decreases during ebb tides the sign of the rheotaxis is reversed and downstream swimming occurs. Thus in nature juveniles will be moving offshore during both flood and ebb tides, swimming in a series of short "hops" against even very strong flood currents (earlier experiments have confirmed this ability) and swimming or being passively displaced with the ebb tide.

Postlarvae within the water column are readily displaced by currents. The rise in salinity occurring with the flood tide causes them to be active in the water column and therefore to be transported inshore. Decreased salinity during the ebb causes them to reduce activity or confine it to deeper layers near the substrate, from where they are better able to withstand the offshore current.

It is not meant to be suggested that salinity changes are the only stimuli inducing appropriate behavioral changes since other aspects of "water quality" may operate similarly. The results do show, however, that salinity changes in the experimental situation elicit responses which, if they are the same in nature, would lead to the type of tide-associated movements which are observed.

That other factors may operate is suggested from records such as those of Champion (pers. comm.) and Slack-Smith (MS) showing that large numbers of juvenile shrimp are present in estuaries (St. Lucia, South Africa and Shark River, Western Australia, respectively) in which the salinities are higher than in the open sea, rising in both to over 60‰. Presumably the shrimp are able to leave these estuaries. It is possible that both postlarvae and juveniles respond similarly to salinities that are either much higher, or lower than normal sea water. If this is the case then rheotaxis of juveniles would be reversed in the presence of increased salinity, causing them to swim downstream during the ebb, and the inshore movement of the postlarvae would be facilitated by an "aversion" to the very high salinities of the ebb similar to that shown to low salinities.

Rainfall—commercial shrimp catch correlations

These results offer a probable explanation for the marked positive correlations reported by Hildebrand and Gunter (1952) and Gunter and Hildebrand (1954) between the commercial catches of *Penaeus setiferus* (L.) off the coast of Texas and the rainfall of the previous year, and similarly between catches of *P. duorarum* and Florida rainfall of the previous year (Iversen, unpublished). Obviously salinity differences between tides would be consistently greater with increase in freshwater runoff. This would facilitate both the movements of postlarvae and juveniles. It is probable that the records of Tabb *et al.* (1962), showing that large juvenile pink shrimp remain in inshore waters when the salinity within the everglades rises to 30‰, are evidence of the breakdown of the tidal transport mechanism in the absence of the usual salinity differences between the tides.

I would like to thank the Committee for Research and Exploration of the National Geographic Society, Washington, D. C., for a grant supporting this work. I am also grateful to Dr. C. P. Idyll for his help and for providing facilities within the Division of Fishery Sciences, University of Miami. My special thanks go to my wife, Philippa, for her invaluable help with the manuscript.

SUMMARY

1. The inshore movements of postlarval pink shrimp and the subsequent offshore movements of the juveniles are facilitated by flood and ebb tides, respectively. This investigation concerns the behavioral mechanisms involved in the selective use of one tide and the evasion of the other.

2. Salinity changes, similar to those occurring with change in tide in the inshore environment usually occupied by pink shrimp, were imposed on both postlarvae and juveniles in a constant-current apparatus.

3. Juvenile shrimp were almost invariably positively rheotactic. However, with a decrease in salinity the sign of the response was reversed, resulting in active downstream swimming. This often gave way to passive drifting.

Under conditions of low light intensity postlarvae were active in the water column, and being unable to withstand even slow currents, were easily displaced. With a decrease in salinity they sank to the substrate or remained low in the water column where they were better able to maintain position.

4. Responses of postlarvae at a discontinuity barrier between bodies of water differing in salinity indicated their ability to perceive differences as small as 1‰. There was an "aversion" to penetrating such a barrier into water of lower salinity.

5. Smaller postlarvae were more "averse" to the barrier than others approximately a week older.

6. If similar responses are elicited in nature during the flood tides, juveniles would orientate and swim against the current in an offshore direction, while postlarvae, by being active in the water column, would be displaced shoreward. Following the decrease in salinity which accompanies the ebb tide the juveniles would swim, or be passively displaced, with the current, again in an offshore direction,

and the postlarvae would sink low in the water column or settle on the substrate from where they are better able to resist displacement.

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SOME SPECTRAL CHARACTERISTICS OF CHLOROPHYLL *c* FROM *TRIDACNA CROCEA* ZOOXANTHELLAE[1]

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Chlorophyll *c* is a photosynthetic pigment which is widely distributed among the marine algae. Although the chemical structure of this pigment has recently been determined (Dougherty *et al.*, 1966), there still seems to be appreciable disagreement in its spectral properties (Jeffrey, 1963; Smith and Benitez, 1955; Strain and Svec, 1966; Mel'nikov and Evstigneev, 1964). Furthermore, Ricketts (1965) found that three different spectroscopic methods of determining chlorophyll *c* (Ricketts, 1965; Parsons and Strickland, 1963; Parsons, 1963) gave very poor agreement. Spectral properties of chlorophyll *c* were investigated in the present study[1] on the biological research vessel *Alpha Helix*, using chlorophyll *c* extracted from dinoflagellate cells (commonly known as zooxanthellae) which were growing symbiotically in the tissues of clams and corals on the Great Barrier Reef. Attention was confined to spectral characteristics of chlorophyll *c* from *Tridacna crocea* zooxanthellae in methanol, acetone and aqueous mixtures of these organic solvents, and their changes with acid and alkali.

Studies carried out on chlorophyll *c* after the 1966 expedition have shown that chlorophyll *c* is a mixture of two spectrally distinct components, designated chlorophyll c_1 and c_2 (Jeffrey, 1968a; Jeffrey, 1968b). Both components are found in chlorophyll *c* isolated from brown seaweeds, diatoms and chrysomonads, but only chlorophyll c_2 has been found in dinoflagellates and cryptomonads. The dinoflagellate character of clam and coral zooxanthellae was established on the 1966 expedition by detailed pigment studies (Jeffrey and Haxo, 1968), and recent tests have shown that the chlorophyll *c* of these symbiotic dinoflagellates consists only of the c_2 component (Jeffrey, 1968b). Furthermore, chlorophyll c_2 isolated from *Tridacna crocea* zooxanthellae showed identical spectral properties to chlorophyll c_2 isolated from other marine algae. The spectral behavior of chlorophyll *c* from *Tridacna crocea* reported here is therefore a study of the universally distributed chlorophyll c_2 component.

EXPERIMENTAL

A number of small rock-boring clams (*Tridacna crocea*) were harvested on Clack Reef on the Great Barrier Reef, and were kept alive in a sea water bath on the research vessel. The dinoflagellate cells were found in very large numbers within the mantle tissue of this clam. The dark brown algal layer was sliced off and homogenized in sea water to obtain a thick brown cell suspension. The suspension was filtered through six layers of cheesecloth to free the cells from tissue debris, and washed three times in filtered seawater by centrifuging at 2,500 g for 5 minutes. The pigments in the cells were readily extracted by first freezing the cells in distilled water for one hour, and then extracting several times with small volumes of methanol until the tissue residue was colorless. The combined methanol extracts were mixed with an equal volume of diethyl ether, and shaken with 10% NaCl solution equal in volume to 10 times that of the methanol-ether extract. The pigments were transferred to the ether layer, and methanol and methanol-soluble impurities were washed into the aqueous phase. The ether layer was then concentrated for chromatography under nitrogen. Full details of the extraction and chromatographic techniques used for pigments in zooxanthellae are described elsewhere (Jeffrey and Haxo, 1968).

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[1] The present study was carried out on the research vessel, R. V. *Alpha Helix*, of University of California during the expedition in 1966 to the Great Barrier Reef, North Queensland, Australia; and was supported by the National Science Foundation of the U. S. A.

Since no materials were available on the research vessel for colume chromatography, purification of chlorophyll *c* was carried out by paper chromatography (Jeffrey, 1961). Pigments were spotted onto 22 cm squares of Whatman No. 3MM paper as a line, and the chromatograms were developed with 4% *n*-propanol in light petroleum (60–80°). The chlorophyll *c* zone ($R_F \simeq 0.2$) was cut out and eluted with either methanol or acetone, depending on which solvent was being used for the study. The chlorophyll *c* obtained was free from all other pigments (chlorophyll *a* and carotenoids), but contained phospholipids, and was therefore equivalent to the Stage 1 chlorophyll *c* in the purification procedure of Jeffrey (1963). The concentrated chlorophyll *c* solution thus obtained was diluted appropriately with the same solvent to prepare a sample solution. Water was mixed with the sample solution when the effect of water was to be examined. Preparation of samples as well as spectroscopic measurements were made at room temperatures near 15°.

Spectroscopic measurements were carried out on the research vessel with a Shimadzu Multipurpose recording spectrophotometer model MPS-50, using 1.0-cm cells. This spectrophotometer, designed by one of the authors, had two photomultipliers, one for the sample and the other for the reference. This made it possible to read a high absorbance value accurately because the interaction (cross talk) between alternative sample and reference signals from a single detector in common recording spectrophotometers was absent in this double detector system. In addition, the photomultipliers had a red-sensitive photocathode of the end-on type. A high resolution was therefore obtained in the spectral region of the red bands of chlorophylls.

Results

Spectral definition of the chlorophyll c preparation

The absorption spectrum of the chlorophyll *c* preparation from *Tridacna* zooxanthellae in methanol is shown in Figure 1, curve A. The Soret and the red bands were located at 451 and 633 mμ and the ratio of Soret to red band was 9.3. A similar measurement was repeated several times with different preparations of chlorophyll *c* at concentrations of 0.84–1.12 in terms of absorbance at the Soret maximum. The average of the band ratios was 9.4 and the standard deviation was 0.06. The band ratio was dependent on the chlorophyll *c* concentration, and was significantly lower when the concentration was doubled. For example, when the absorbance at the Soret maximum was 1.92, the band ratio was 8.7. If this may

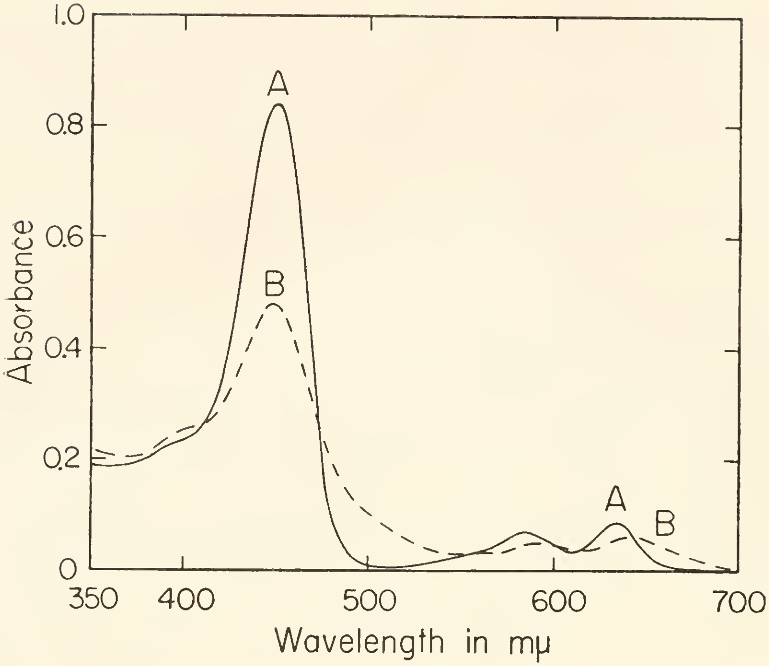

FIGURE 1. Absorption spectrum (curve A) of chlorophyll *c* in absolute methanol as compared with that (curve B) in 1:3 methanol-water mixture at pH 7.3. The concentrations of chlorophyll *c* in these measurements were identical.

be interpreted as due to polymerization of chlorophyll *c* molecules, the higher ratio, 9.4, obtained at lower concentrations may be the more correct ratio for monomeric chlorophyll *c* in the particular preparation studied.

The spectrum of the chlorophyll *c* preparation in acetone showed Soret and red maxima at wavelengths (453 and 628 mμ) slightly different from those in methanol. The band ratio was 9.4, being identical with the ratio obtained with methanol at lower chlorophyll *c* concentrations. The ratio determined previously (Jeffrey, 1963) with acetone immediately after purification of chlorophyll *c* was 9.3 while the ratio decreased to 6.9–8.1 during storage for more than 2 hours at − 20°. The present result confirmed the previous value obtained immediately after purification. The flattening of the Soret band after purification might have been due to aggregation of chlorophyll molecules, made possible by the removal of the last traces of lipid from the preparation and storage at low temperatures. Similar flattening effects of the Soret band were found in the present work by the addition of water to acetone or methanol solutions of chlorophyll *c*.

An example of the effect of water on the absorption spectrum of chlorophyll *c* is shown by curve A in Figure 2, which shows the spectrum of chlorophyll *c* in a 5:1 methanol mixture (17% water by volume) at an apparent pH of 7.4. On the addition of water, the Soret maximum shifted to a shorter wavelength of 448 mμ, but the red maximum did not alter (634 mμ). The band ratio fell to 8.2. This ratio was confirmed by repeated measurements, and was significantly lower than the ratio of 9.3 obtained with absolute methanol. The apparent pH values of the mixtures in these measurements were between 7.0 and 8.3, and the measurements were made immediately after the addition of water to the chlorophyll *c* solution in absolute methanol, although the spectrum did not appreciably change later. Curve B in Figure 1 is the spectrum of the same concentration of chlorophyll *c* in a 1:3 methanol-water mixture (75% water). With such a large content of water, the Soret band decreased remarkably to about 60% of the height in absolute methanol, and the red band to about 70% of the original height with a shift to 642 mμ. The band ratio under this condition was 8.0. A similar effect of water was found with acetone as solvent. The Soret and the red bands in the 9:1 acetone-water mixture (10% water) were at 448 and 628 mμ, respectively, and the band ratio was 8.7 (curve A, Fig. 3). A more pronounced drop of the Soret band was found when a salt solution or a buffer was added in place of distilled water to cholorophyll *c* in methanol or acetone: the higher the salt concentration, the lower the Soret band. The wavelengths and the relative heights of absorption maxima in these different solvents are summarized in Table I together with the data obtained with acid and alkali.

Spectral changes with acid

The effect of acid on the spectrum of chlorophyll *c* was observed with 5:1 methanol-water mixtures (17% H_2O). The spectrum of chlorophyll *c* in the mixture at pH 7.4 as the control was first measured (curve A, Fig. 2), and a minute

TABLE I

Absorption maxima and relative band heights of chlorophyll c from Tridacna *zooxanthellae and derivatives formed by acid and alkali treatments*

Solvent	λ_{max} in mμ (relative band heights)
Methanol	451 586 633 (9.4:0.80:1.00)
Methanol-water (5:1)	448 584 633 (8.1:0.70:1.00)
Methanol-water (1:3)	448 592 642 (8.0:0.83:1.00)
Methanol-water (5:1) + NaOH	425 561 600 (29.0:1.96:1.00)
Methanol-water (5:1) + NH_4OH	426 564 604 668 (20.9:1.64:1.04:1.00)
Acetone	453 583 628 (9.4:1.06:1.00)
Acetone-water (9:1)	448 582 628 (8.7:0.83:1.00)
Acetone-water (9:1) + HCl	431 532 574 596 (13.0:0.93:1.03:1.00)

volume of a dilute HCl solution on the fine tip of a spatula was added to the solution. The spectrum was measured immediately after the addition as well as later at intervals. The spectrum of the solution at pH 3.5 obtained immediately after the acidification is shown by curve B in the same figure which indicates the Soret band at a longer wavelength of 452 mμ with a great drop of the height. The red band was shifted also to a longer wavelength of 638 mμ with an appreciable decrease of height. A qualitatively different change started 5 minutes after the acidification, and the Soret band, which was once shifted to the longer wavelength, was shifted to the opposite direction to a greater extent with continuous lowering of band. The band positions observed at 5, 15, and 30 minutes after the acidification were 447, 433, and 428 mμ, respectively. The spectrum obtained after 30 minutes of incubation is shown by curve C. The Soret band obtained after 100 minutes (curve D) was at 435 mμ, and practically no change was observed later.

The spectral change by acid observed with acetone as the organic solvent was different from this change in methanol. In the experiment, a minute volume of a HCl solution was added to a neutral solution of chlorophyll *c* in 9:1 acetone-water (10% H_2O) mixture (curve A, Fig. 3) to prepare an acidic solution (curve B), and the measurement was made within 5 minutes after the acidification, although the spectrum did not change later. Curve B obtained at the strongly acidic pH of 0.6 indicates a sharp Soret band at 431 mμ and three bands at 532, 574, and 596 mμ. This contrasts with the remarkable drop of the Soret band by acid in methanol.

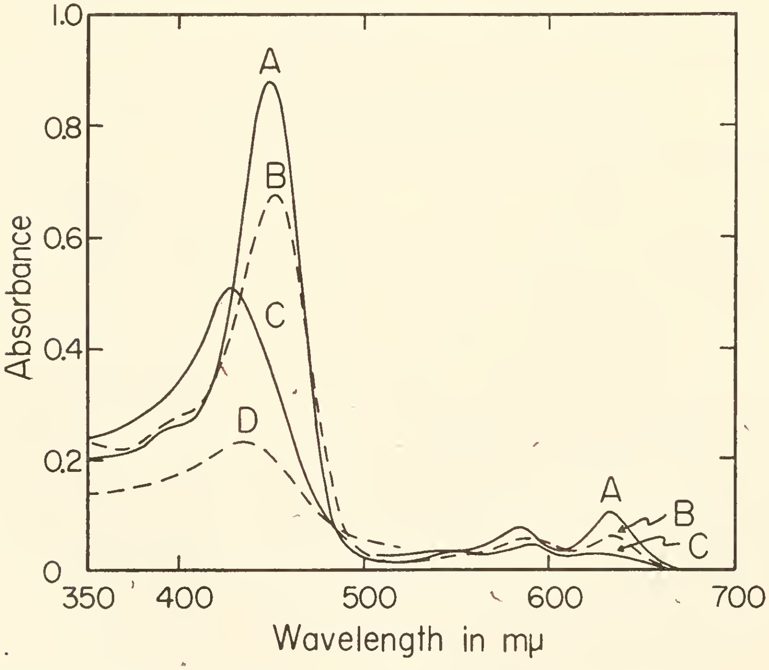

FIGURE 2. Absorption spectrum (curve A) of chlorophyll *c* in 5:1 methanol-water at pH 7.4, and the spectra of the same solution at pH 3.5 mixed with a minute volume of HCl and observed immediately (curve B), 30 minutes (curve C), and 100 minutes (curve D) after the acidification.

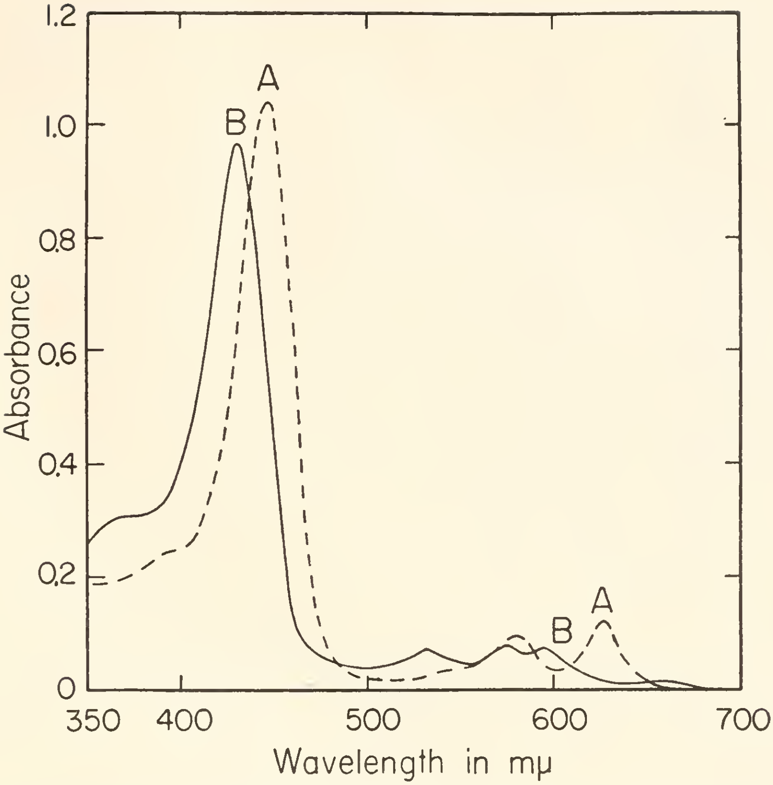

FIGURE 3. Absorption spectrum (curve A) of chlorophyll *c* in 9:1 acetone-water mixture at pH 7.8 and the spectrum (curve B) of the same solution at pH 0.6 acidified with a minute volume of HCl.

Curve B shows a very weak band around 660 mμ, and its height is less than 20% of the red band at 595 mμ. This may be either a band of pheophytin *c* or a band of an impurity which was masked by the red band of chlorophyll *c* before the treatment with acid.

Spectral changes with alkali

Curve A in Figure 4 is the spectrum of chlorophyll *c* in 5:1 methanol-water mixture of pH 8.3 as the control, and curve B is the spectrum of the same solution after addition of a small volume of a concentrated NaOH solution. The apparent pH value of this alkaline mixture was 13.4, and the spectrum was observed 5 minutes after the addition of alkali. The Soret band was transformed with alkali into a band at 425 mμ with great intensification, and no further change took place after 5 minutes of incubation. The intensification was as much as 1.9 times. Such a spectral change was observable above pH 12, although the band transformation did not proceed to completion below pH 12.5 within a limited time of incubation. It is to be noted that a clear isobestic point was observed at 437 mμ in the transformation of the Soret band. The two red bands of chlorophyll *c* disappeared on the treat-

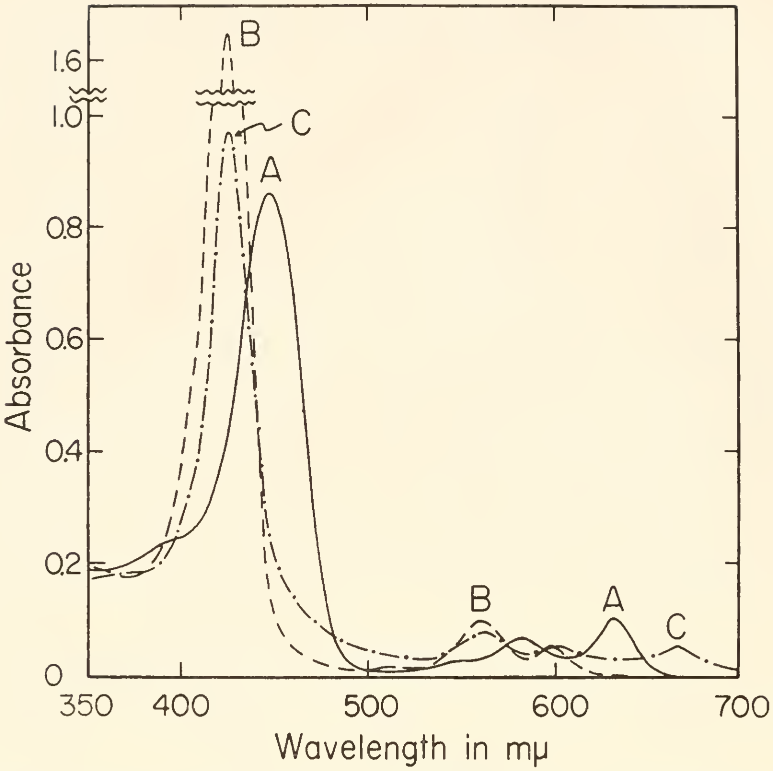

FIGURE 4. Absorption spectrum (curve A) of chlorophyll *c* in 5:1 methanol-water mixture (pH 8.3) as the control, and the spectrum (curve B) of the same solution at pH 13.4 mixed with a minute volume of a concentrated NaOH solution and observed 5 minutes after the addition of alkali. Curve C is the spectrum of a different concentration of chlorophyll *c* in a mixture (pH 12.0) of absolute methanol and 28% aqueous ammonia in a volume ratio of 5:1. Curve C was observed 250 minutes after the addition of aqueous ammonia.

ment with alkali and were replaced by new bands at 561 and 600 mμ. Isobestic points existed at 575, 595 and 612 mμ in this transformation. These spectral changes may therefore, be interpreted as due to a simple transformation from one compound to another.

The spectral change with ammonia at alkaline pH was found to be different from this change with NaOH. One ml of 28% aqueous ammonia was added to 5 ml of a chlorophyll *c* solution in methanol, and the spectrum of the mixture was measured at intervals. A different spectral change proceeded slowly during 4 hours of incubation with ammonia at pH 12.0, and the resultant spectrum shown by curve C in Figure 4 indicated the Soret band at 426 mμ and three bands at 564, 604, and 668 mμ, respectively. There seems to be another band near 630 mμ, considering the high and flat absorption curve between the two separate bands at 604 and 668 mμ. The two bands at 564 and 604 mμ are similar in position and in the relative height to the visible bands of NaOH-treated chlorophyll *c* (curve B), so that the band at 668 mμ may be characteristic of this ammonia-treated chlorophyll *c*. The intensification of the Soret band by ammonia was estimated to be 1.6 times which is less than the ratio obtained with NaOH. Chlorophyll *c* may, therefore, be transformed with ammonia into a compound which is different from that derived with NaOH.

Discussion

Chlorophyll *c* prepared from *Tridacna crocca* zooxanthellae, and now known to be the recently described chlorophyll c_2 component (Jeffrey, 1968a; Jeffrey, 1968b) had a ratio of Soret band to 630 mμ band in absolute methanol and absolute acetone of 9.4. This agrees with recent data obtained for chlorophyll c_2 isolated from other groups of algae (Jeffrey, in preparation). A most important finding in the present work was the loss of intensity of the Soret band of chlorophyll *c* in organic solvents by the addition of water, while the red band was much less affected by the addition. The decrease was obtained more rapidly and to a greater extent, (a) at higher water contents or higher salt concentrations in the solvent, and (b) at higher chlorophyll *c* concentrations. In previous work (Jeffrey, 1963) a decrease of the band ratios occurred at, (c) low temperatures, or (d) when the lipid content of the sample was low. This spectral change is interpreted from these facts as due to aggregation or polymerization of chlorophyll *c* molecules. The band ratio of 9.4 determined at low chlorophyll *c* concentrations in absolute methanol or acetone in the present preparation can be taken as an expression of the proportion of the monomeric species of chlorophyll c_2 in the solution.

When chlorophyll *c* in aqueous methanol was acidified, the Soret band dropped remarkably, and the drop was accompanied first by a slight shift of band toward longer wavelengths, and second by a greater shift toward shorter wavelengths. Similarly two steps of band shift with continuous drop of band were found when an alkaline solution of monomeric hematin was neutralized by addition of distilled water or a HCl solution (Inada and Shibata, 1962). The kinetic analysis of this spectral change suggested that the first drop of band with a shift to longer wavelengths is due to dimerization and the second drop with a shift to shorter wavelengths is due to further polymerization of dimeric hematin. The same interpretation may be given from the similarity to the two steps of the Soret band change of chlorophyll *c*. This view may be supported by the recent finding of Mel'nikov and Evstigneev (1964) that chlorophyll *c* evaporated in ether to dryness under a stream of hot air and dissolved in ether, benzene or toluene shows an additional band on the longer wavelength side of the Soret band and the red band at 665 mμ. These authors ascribed the appearance of these bands to association of chlorophyll *c* molecules. Another fact found by them is that the spectrum of the associated form changed back to the original spectrum when several drops of water were added to the ether solution. This appears to conflict with our observation that the Soret band drops to a greater extent at higher water contents in the medium. The different results may, however, result from the difference in the solvent used between these experiments. When chlorophyll *c* was acidified in acetone as the solvent, the Soret band was transformed into a sharp band at 431 mμ which may be interpreted as a band of pheophytin *c*. The two red bands of chlorophyll *c* disappeared on the treatment and were replaced by three bands at 532, 573, and 595 mμ.

The Soret band of chlorophyll *c* was greatly intensified and shifted to 426 mμ by alkaline hydrolysis with NaOH. Clear isobestic points were observed in this process, indicating that polymerization is not involved in this change. The treatment with ammonia at alkaline pH gave a different spectrum. The chemical changes by NaOH and by ammonia have to be worked out based on the structures of chlorophyll *c* recently determined.

SUMMARY

Absorption spectra of chlorophyll *c* prepared from *Tridacna* zooxanthellae (and now known to be the component chlorophyll c_2) were scrutinized with special attention to the ratio of the Soret to the red band in methanol and acetone and the spectral changes resulting from the addition of water, acid and alkali. The band ratios of the chlorophyll *c* preparation determined with methanol and with acetone as the solvent were 9.4, but the ratios determined in aqueous mixtures of these organic solvents were appreciably lower than this value, which was interpreted as due to polymerization of chlorophyll *c* molecules. When chlorophyll *c* in aqueous acetone was acidified with HCl, the spectrum was changed to a spectrum with a sharp Soret band which is regarded as the spectrum of pheophytin *c*. When acidified in aqueous methanol, however, the Soret band dropped remarkably, being accompanied first by a slight shift of band to longer wavelengths and next by a greater shift to shorter wavelengths. These two steps of band shifts were interpreted as due to dimerization and further polymerization of dimeric chlorophyll *c,* respectively. The alkaline hydrolysis with NaOH and that with ammonia gave different spectra. These spectral characteristics of chlorophyll *c,* isolated from *Tridacna crocea* zooxanthellae, will help to resolve controversies over the spectral properties of this pigment, especially those caused by unrecognized polymerization.

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OSMOREGULATION IN A MARINE CILIATE, *MIAMIENSIS AVIDUS*. I. REGULATION OF INORGANIC IONS AND WATER[1]

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Little is known of the content and regulation of inorganic ions in marine protozoa. The ionic composition of *Uronema filificum* (Thompson and Kaneshiro, 1968), a marine ciliate, has been determined (Kehlenbeck *et al.*, 1965) and certain aspects of contractile vacuole function have been studied in several forms (Kitching, 1938, 1967).

Miamiensis avidus (Thompson and Moewus, 1964), a euryhaline ciliate, can be studied after exposure to large changes in environmental osmolarity. Also, it grows axenically in mass cultures and has a short generation time, so chemical analyses are feasible. Taking advantage of these features, we have sought information on the function of the contractile vacuole and on the kinds and concentrations of internal inorganic ions.

Materials and Methods

Miamiensis avidus, a facultative parasite of seahorses was obtained from Dr. Liselotte Moewus and maintained axenically in a medium of the following composition: lactalbumin hydrolysate solution (10%, w/v) (Nutritional Biochemicals Corp.) 10 ml, calf serum (Grand Island Biological Co.) 5 ml, filtered sea water 85 ml. All components were sterilized separately and combined aseptically. The complete medium was adjusted to pH 7.5 with sterile 4.2% $NaHCO_3$ (Moewus, 1963).

Intracellular inorganic ion analyses

One-liter mass cultures were grown in 2500-ml low-form Erlenmeyer flasks at 24–26° C. A 20-ml inoculum of a log phase culture was introduced into each flask. Cells in late log phase to early stationary phase were concentrated by centrifugation in 40-ml conical tubes at 300 *g* for 3 min and then exposed to various experimental conditions.

Appropriate concentrations of artificial sea water (100% = 31‰), a solution composed of the major salts found in sea water (Marine Biological Laboratory Formulae and Methods V., 1964), were added to cell suspensions to produce high osmolarity test environments. Distilled water was added for low osmolarity environments. Sucrose (0.72 *M*) isosmotic to M.B.L. sea water was used in some experiments to maintain osmotic pressure when inorganic ion concentrations were being varied. In ion substitution experiments, Na^+, K^+, and Ca^{++} were replaced by choline$^+$. Mg^{++} was replaced by Na^+, and Cl^- was replaced by proprionate$^-$ and SO_4^{--}.

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C^{14}-inulin was added to cell suspensions within 2 min prior to centrifugation (1000 *g* for 5 min) for estimation of extracellular space (Dunham and Child, 1961). An extracellular space of 25%, based on a mean of 106 determinations, was used in all calculations of cellular ion content. Duplicate 0.1-ml aliquots of cell suspensions were preserved for counting cell numbers in a Sedgewick-Rafter chamber with the aid of a Whipple ocular micrometer. Cell volume was calculated using the equation:

$$\text{volume/cell} = \frac{\text{(wet wt of pellet) (1-inulin space)}}{\text{no. cells/10 ml cell suspension}}$$

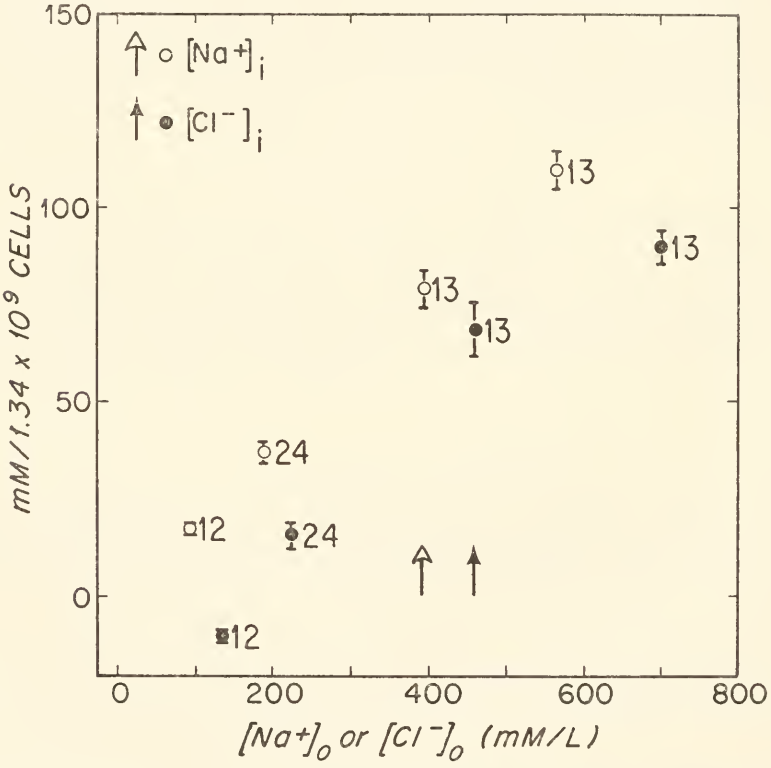

FIGURE 1. Changes in $[Na^+]_i$ and $[Cl^-]_i$ of *M. avidus* (expressed as m*M*/initial liter of cells) following changes in $[Na^+]_o$ and $[Cl^-]_o$. In this and the following figures, ion concentrations are expressed in m*M*/original liter of cells, *i.e.*, expressed in terms of the number of cells in the initial control liter of cells (kg cells), since the volumes of cells change under various experimental conditions. Means, standard errors of the means, and number of determinations are indicated. Arrows show values in 100% sea water.

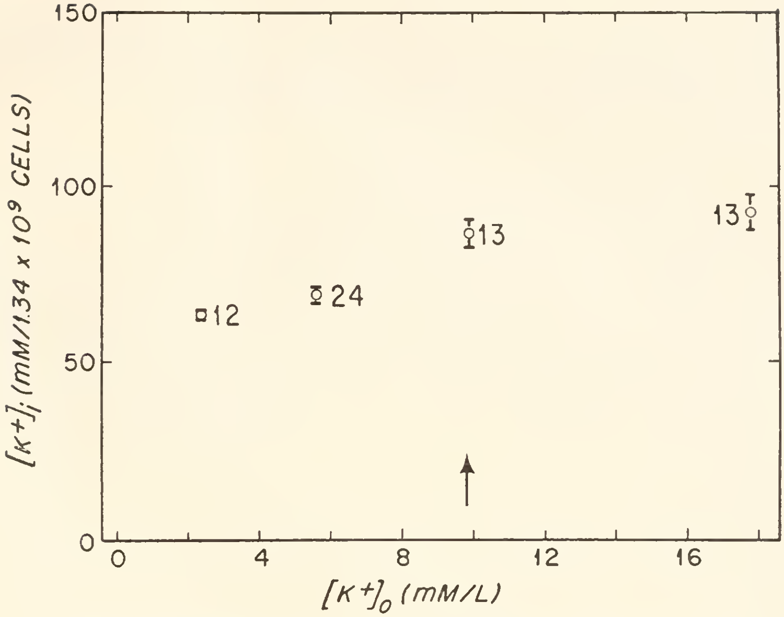

FIGURE 2. Changes in $[K^+]_i$ of *M. avidus* following changes in $[K^+]_o$.

Inorganic ions were extracted in 10 ml of dilute acetic acid. The extraction tubes were placed in a 100° C water bath and allowed to stand for at least an hour. After extraction, the debris was packed by centrifugation and the supernatant decanted. Values obtained for the ion contents of ciliates were corrected for extracellular space and cell volume changes.

Na^+, K^+, Ca^{++} and Mg^{++} were determined with a Coleman photometer and Cl^- by electrometric titration (Aminco-Cotlove titrator).

Contractile vacuole output

Frequency of vacuole pulsation in different solutions was observed directly with a phase contrast microscope.

To compare the vacuole output in different test solutions, ciliates were equilibrated for more than 30 min and were observed and photographed within 5 hours. Time-lapse photographs (Sage Cinephotomicrographic Apparatus, Model 500, Bolex H16 M camera) were taken at 60 frames/min and 420 × magnification. Nothing was done to immobilize the ciliates, and those that showed signs of compression by the cover slip (vesiculated cytoplasm or everted oral area) were not included in the measurements. A stage micrometer was filmed for calibration purposes. Cell length and width, and contractile vacuole diameter were measured with the aid of an analytical movie projector. For calculation of cell volume the shape of the vacuole was considered to be a sphere, and the shape of the ciliate to be a cone plus a hemisphere. Values for cell volume obtained by this method agreed with those described above.

Vacuolar rate was expressed as volume output/unit time. Volume output/unit time was divided by the cell volume to give the fraction of cell volume turnover/unit time. Visual observations and photography were completed within 5 min after slides of equilibrated ciliates were prepared for examination.

RESULTS

Intracellular inorganic ion concentration in Miamiensis avidus

When the intracellular concentrations of inorganic ions were determined in ciliates from 100% sea water, and from various concentrations above and below 100% 30 min after a change in the external salinity, it was found that intracellular Na^+ ($[Na^+]_i$) and Cl^- ($[Cl^-]_i$) were lower than environmental values ($[Na^+]_o$, $[Cl^-]_o$)[5] (Fig. 1). They increased as external salinity was increased and decreased as external salinity was decreased, but always remained lower than the environment. The ciliates maintained $[Na^+]_i$ at ⅕, and $[Cl^-]_i$ at about $\frac{1}{7}$ the environmental values at all sea water concentrations tested. Since $[Cl^-]_i$ is very low relative to $[Cl^-]_o$, estimates of $[Cl^-]_i$ are particularly subject to large variability with slight errors in determinations of extracellular space. The negative value for $[Cl^-]_i$ in 25% sea water might be accounted for in these terms.

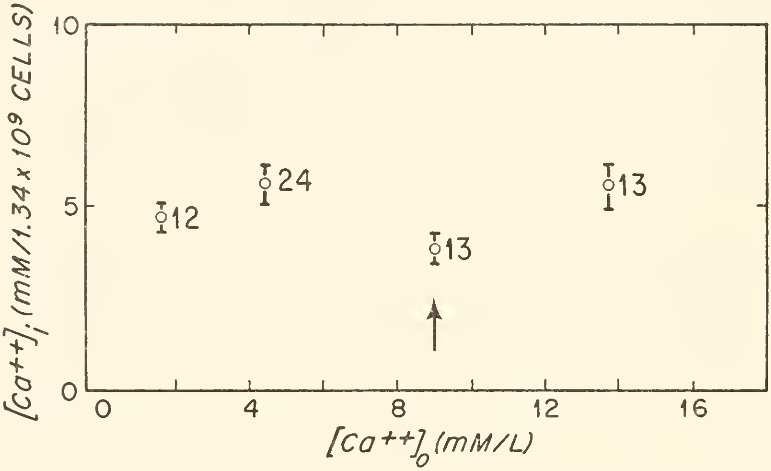

FIGURE 3. Changes in $[Ca^{++}]_i$ of *M. avidus* following changes in $[Ca^{++}]_o$.

$[K^+]_i$ was considerably higher than $[K^+]_o$ in the ciliates from 100% sea water and remained at a fairly constant concentration over the range of external salinity changes tested (Fig. 2). Atypical of most cells, ciliates in 100% and 150% sea water had $[Na^+]_i$ greater than $[K^+]_i$ since $[Na^+]_i$, but not $[K^+]_i$, increased as external salinity was increased. $[K^+]_i$ was greater than $[Na^+]_i$ in ciliates in 50% and 25% sea water.

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[5] $[\ \ ]_i$—intracellular concentration (m*M*/original liter cells); one liter cells was taken to be equivalent to one kg cells.
$[\ \ ]_o$—extracellular concentration (m*M*/liter).

TABLE I

Intracellular inorganic ion concentrations of M. avidus cultured and equilibrated in sea water-culture media

% Sea water-culture medium (exposure time)	mM/kg cells*			
	[±1.4 – 2.7 mM (SE)]			
	$[Na^+]_i$	$[K^+]_i$	$[Cl^-]_i$	N**
100% (2 years)	87.9	73.7	60.8	104
50% (3 months)	52.8	60.6	24.9	31
100% (2 years) 50% (30 min)	37.1	69.8	16.0	24

*mM/1.44 × 10^9 cells (1 liter).
** No. determinations.

$[Ca^{++}]_i$ and $[Mg^{++}]_i$ were lower than $[Ca^{++}]_o$ and $[Mg^{++}]_o$ in ciliates from 100% sea water and remained constant despite changes in salinity (Figs. 3, 4). Internal concentrations of these ions were, therefore, higher than the external concentrations when the ciliates were in diluted sea water and lower than external concentrations when they were in 150% sea water.

When the external environment was diluted by one-half (to 50% sea water) or made more concentrated (to 150% sea water), and the inorganic ion content of the ciliates analyzed at different times thereafter, the net movements of monovalent ions (Na^+, Cl^- and K^+; Figs. 5, 6, 7) were essentially completed about 10 min after exposure to the new conditions. There were no net movements of Ca^{++} and Mg^{++}.

Ion concentrations of ciliates subcultured for 2 years in 100% sea water-culture medium were also compared with the following: ciliates subcultured for 3 months

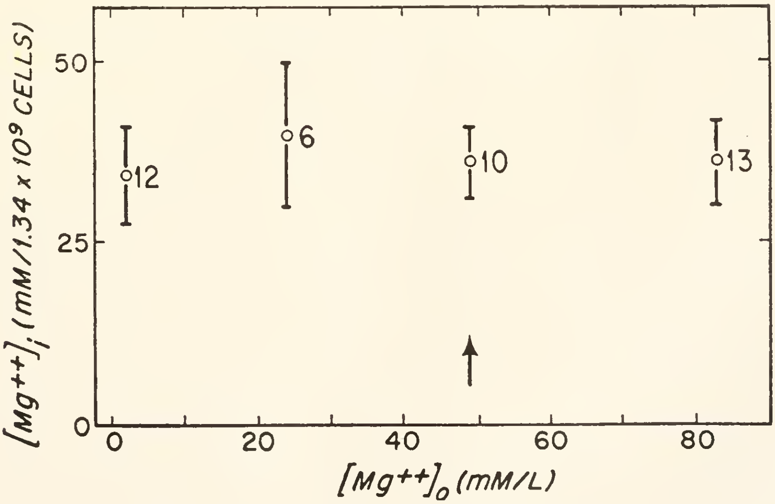

FIGURE 4. Changes in $[Mg^{++}]_i$ of *M. avidus* following changes in $[Mg^{++}]_o$.

TABLE II

Intracellular inorganic ion concentrations of cultured U. filificum

% Sea water-culture medium	mM/kg cells [±2.3 – 9.4 mM (SE) in 22 determinations each ion]		
	$[Na^+]_i$	$[K^+]_i$	$[Cl^-]_i$
100%	84.4	134.1	16.1
50%	63.8	102.9	1.7

in 50% sea water-culture medium and then equilibrated in 50% sea water-culture medium for 30 min (Table I). $[Na^+]_i$ and $[Cl^-]_i$ were lower in ciliates subcultured in 50% sea water-culture medium than in ciliates subcultured in 100% sea water-culture medium. They were higher in ciliates subcultured in 50% sea water-culture medium than in ciliates subcultured in 100% sea water-culture medium and then equilibrated in 50% sea water-culture medium for 30 min. $[K^+]_i$ and $[Ca^{++}]_i$ were about the same under all three conditions. $[Mg^{++}]_i$ was not determined.

Intracellular inorganic ion concentrations in Uronema filificum

Preliminary results on the inorganic ion content of a free-living, marine ciliate, *Uronema filificum,* were reported earlier (Kehlenbeck *et al.,* 1965). For comparative purposes more complete data are shown in Table II. As in the case of *M. avidus, U. filificum* had a greater $[K^+]_i$ than $[K^+]_o$ in 50% and in 100% sea water while $[Na^+]_i$ and $[Cl^-]_i$ were lower than $[Na^+]_o$ and $[Cl^-]_o$. *U. filificum* had a greater $[K^+]_i$ and a lower $[Cl^-]_i$ than *M. avidus.*

TABLE III

Intracellular inorganic ion concentrations of M. avidus *equilibrated for 30 min in various test solutions*

	mM/kg cells [±2–6 mM (SE) in 3–6 determinations]		
	$[Na^+]_i$	$[K^+]_i$	$[Cl^-]_i$
100% Sea water	92.3	78.3	91.5
50% Sea water	48.6	61.5	31.9
50% Sea water 0.72 M Sucrose	85.5	56.7	93.5
100% Sea water 50% Na^+	53.6	82.1	98.9
100% Sea water 50% Cl^-	108.7	81.7	60.1

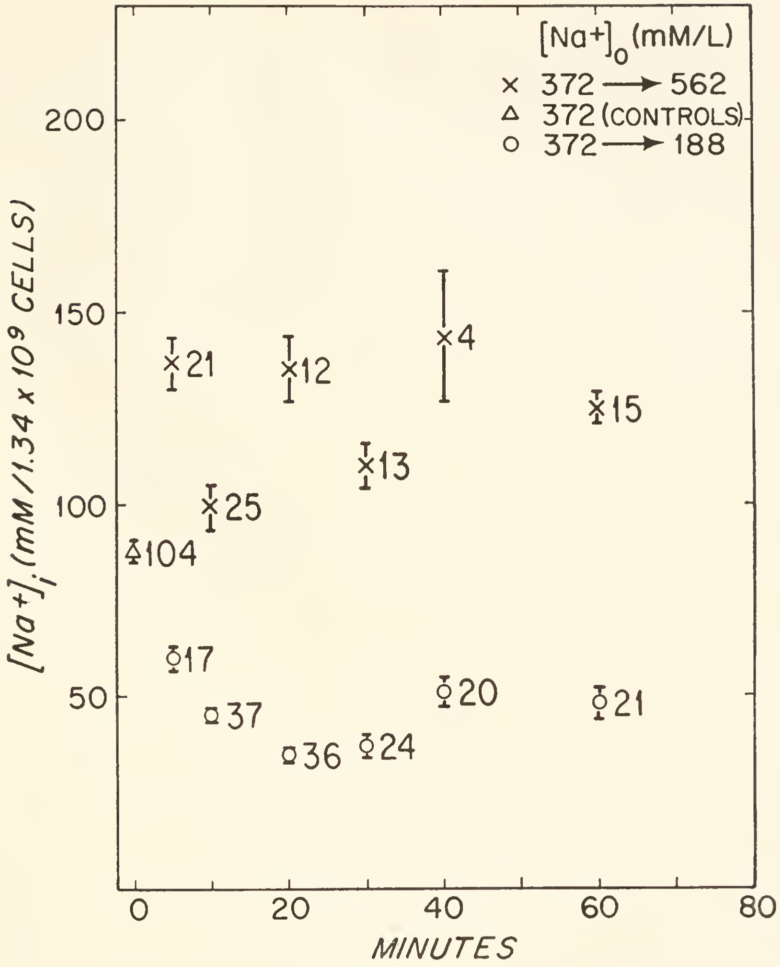

Figure 5. Net influx and net efflux of Na^+ in *M. avidus*.

Ion substitution experiments

The effects of ion substitutions on intracellular inorganic ion concentrations were analyzed 30 min after *M. avidus,* subcultured in 100% sea water-culture medium, was equilibrated in test solutions (Table III). When 50% of the Na^+ in the final test solution was replaced with choline$^+$, $[Na^+]_i$ decreased ($p < 0.001$); however, $[K^+]_i$ was unaffected by a 50% reduction in $[K^+]_o$ replaced by choline$^+$. $[Cl^-]_i$ was reduced when propionate$^-$ and SO_4^{--} replaced 50% of the Cl^- in the external solution ($p < 0.001$). $[Ca^{++}]_i$ and $[Mg^{++}]_i$ were not altered by any of the external changes in ion composition or ion concentration tested. When sucrose isosmotic to sea water was substituted for 50% of the sea water, the intracellular concentrations of all ions except K^+ were maintained at the level found in cells in 100% sea water. $[K^+]_i$ was reduced ($p < 0.001$).

Contractile vacuole output

In *M. avidus* the fraction of cell volume eliminated by the contractile vacuole per unit time increased with decreasing external osmotic pressure (Fig. 8). On transfer to media of osmolarity greater than sea water, there was a slight decrease in the rate of fluid output by the vacuole. In 25% sea water the contractile vacuole expelled an amount of fluid equal to the cell volume in about a half-hour; in 175% sea water in about 2 hours. That the contractile vacuole output rate was responsive to changes in external osmolarity and not ionic strength was shown when the external salt concentration was decreased by 50% but the osmotic pressure was maintained at the value for 100% sea water by the addition of sucrose. In this experiment the average fraction of cell volume turnover/min was 0.011 (± 0.003, SE; 11 determinations). The output rates of ciliates in 50% sea water and in the sucrose-sea water solution were significantly different ($p < 0.05$).

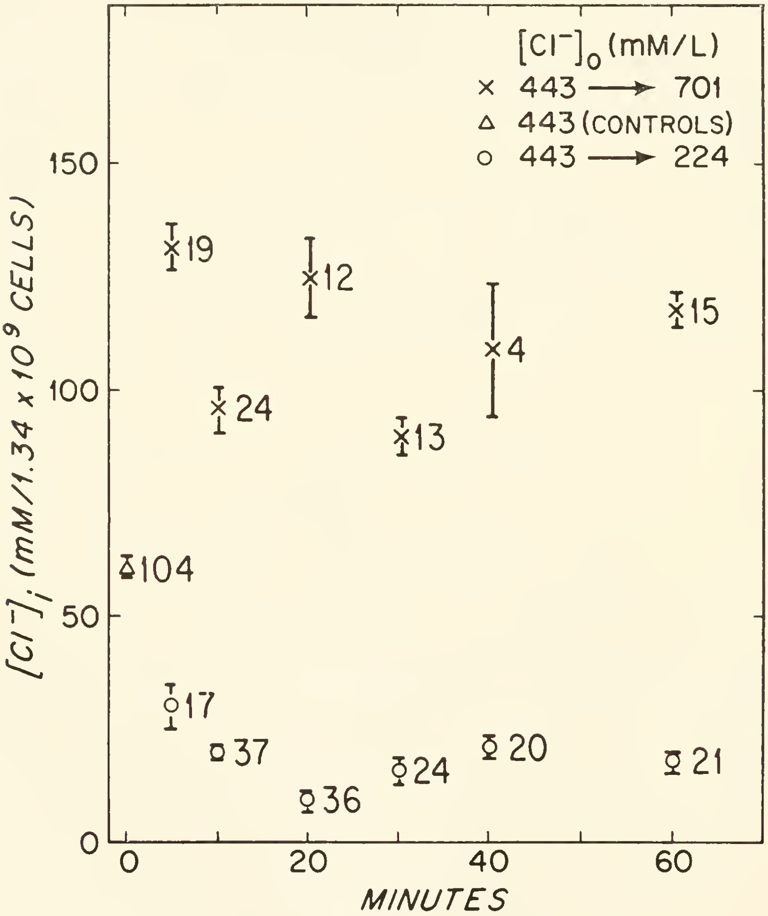

FIGURE 6. Net influx and net efflux of Cl^- in *M. avidus*.

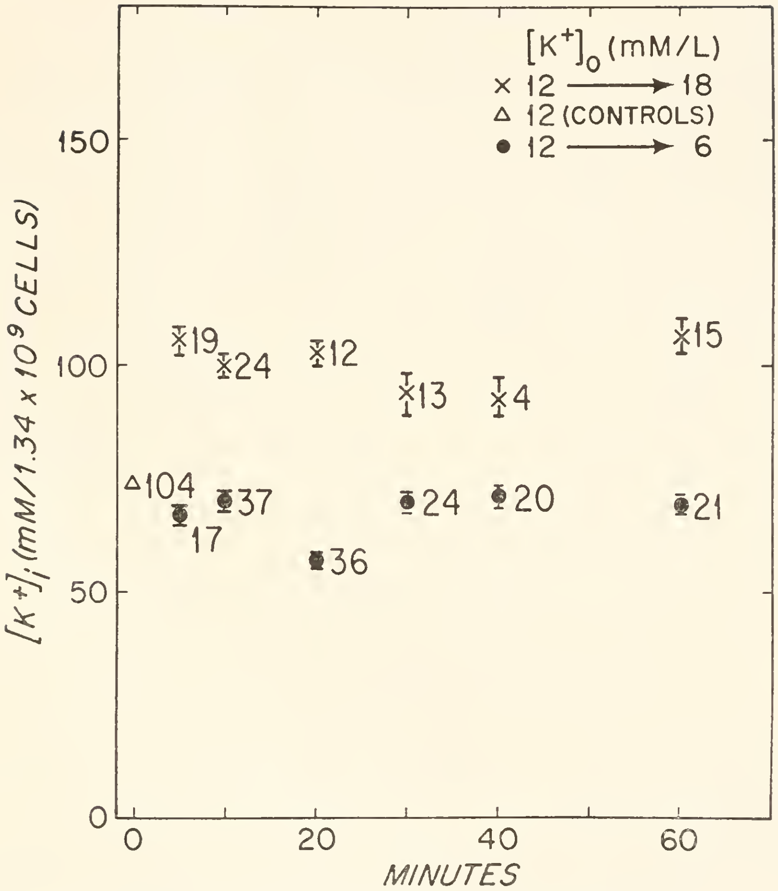

Figure 7. Net influx and net efflux of K^+ in *M. avidus.*

Cell volume

When exposed to environments more dilute or more concentrated than sea water, *M. avidus* swelled or shrank, respectively. Figure 9 illustrates the volumes of ciliates equilibrated for 30 min in media of different salinities. After being in the new environment for about 90 min, the ciliates almost regained their original volumes (Fig. 10).

Discussion

For both *M. avidus* and *U. filificum* the concentration gradients of K^+, Na^+ and Cl^- between the ciliates and the environment were typical for animal cells. The mechanisms responsible for the maintenance of the gradients are not known. If the cytoplasm is electrically negative to the external medium, the Na^+ gradient is far from thermodynamic equilibrium. Therefore active transport of Na^+ is likely since the ciliates are permeable to Na^+. The contractile vacuole may be responsible for

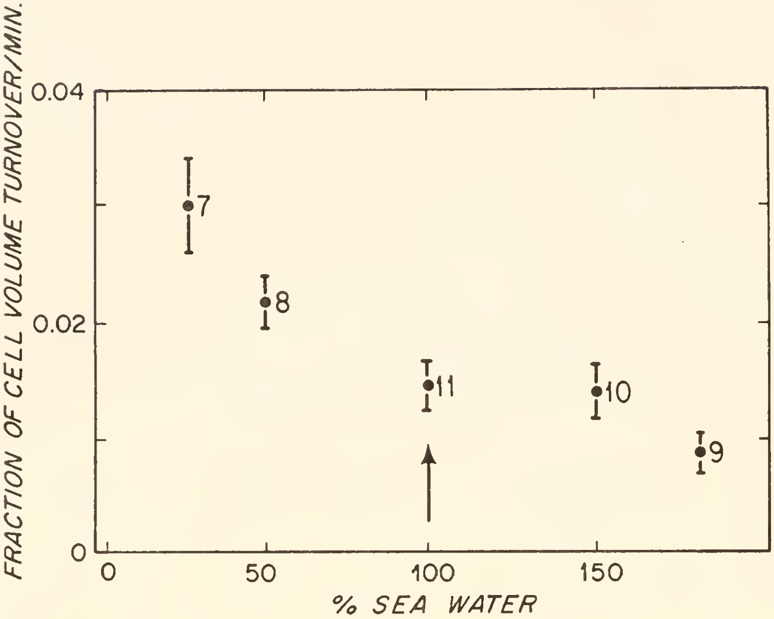

FIGURE 8. Vacuolar output of *M. avidus* (expressed as fraction of cell volume turnover/min) as a function of sea water concentration.

active extrusion of Na^+ (Chapman-Andresen and Dick, 1962; Marshall, 1966; Dunham and Stoner, 1967). Maintenance of $[K^+]_i$ may be passive or may depend on active accumulation as in some fresh water protozoa (Dunham and Child, 1961; Conner, 1967).

M. avidus placed in 50% sea water with sufficient sucrose to make it isosmotic to 100% sea water had $[Na^+]_i$ and $[Cl^-]_i$ at the levels found in ciliates in 100% sea water, *i.e.*, significantly higher than in ciliates in 50% sea water. $[Na^+]_i$ and $[Cl^-]_i$ are, therefore, functions of the external osmolarity and not of ionic strength. However, the decrease in $[Na^+]_i$ in response to propionate$^-$ and SO_4^{--} substitutions in the medium are not easily reconciled with the maintenance of normal $[Na^+]_i$ and $[Cl^-]_i$ in 50% sea water + sucrose.

The relationship between the rate of fluid output by the contractile vacuole and the osmolarity of the external medium suggests an osmoregulatory function for the contractile vacuole. Kitching (1936) also observed an increased rate of contractile vacuole output in marine peritrich ciliates upon transfer to dilute sea water but did not measure output rates in sea water concentrations above 100%. The continued function of the vacuole of *M. avidus* in all sea water concentrations tested, including 200%, suggests that the cells are hyperosmotic to all of the media. Since vacuolar output was only slightly reduced after transfer to media more concentrated than 100% sea water, the intracellular osmolarity must increase after the transfer, such that the difference between intracellular and external osmolarities does not change much. On the other hand, the contractile vacuole may be removing water from a source other than osmotic entry, such as water derived from metabolism.

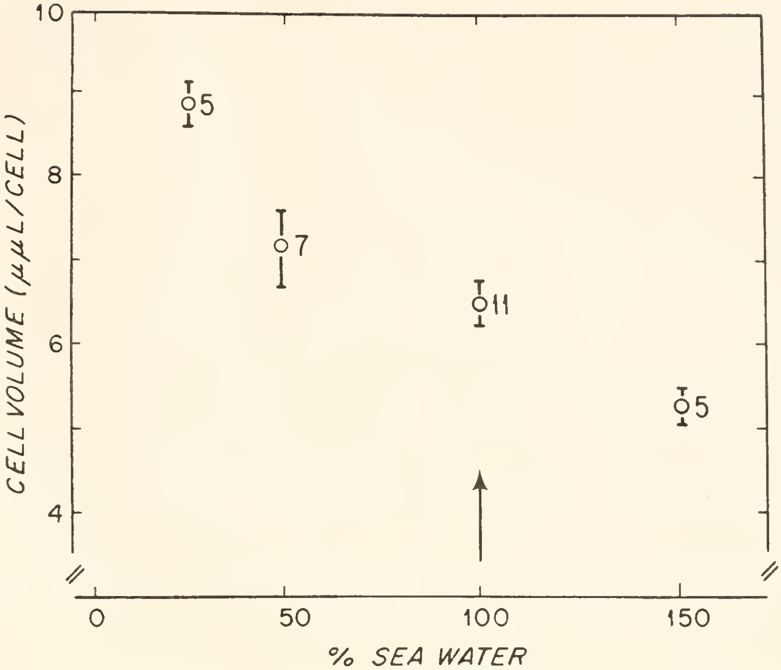

FIGURE 9. Changes in cell volume of *M. avidus* equilibrated for 30 min in different concentrations of sea water.

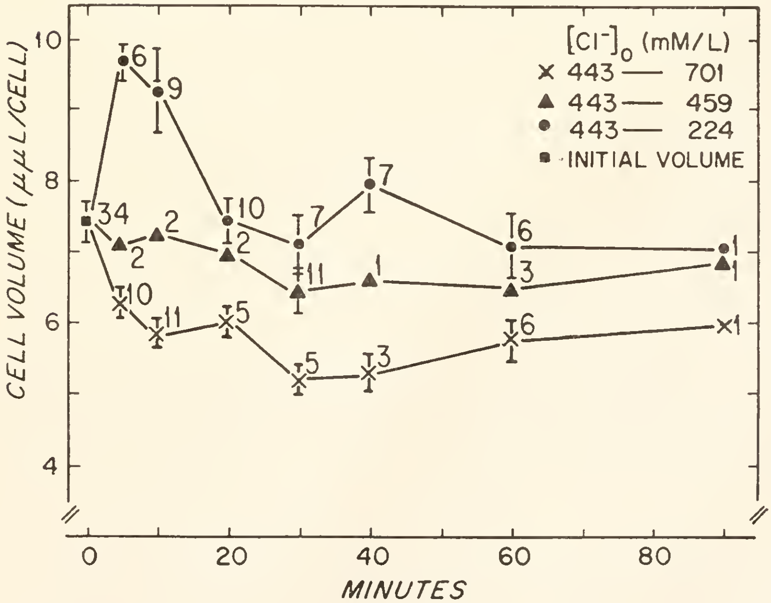

FIGURE 10. Cell volumes of *M. avidus* at different times after transfer to different salinities.

In fresh-water protozoa, metabolic water is a minor fraction of vacuolar output (Kitching, 1967); however, metabolic water might be a significant aspect of water balance in marine protozoa.

After osmotic challenges, cell volume changed passively, but then returned toward the volume of ciliates kept in 100% sea water (Fig. 10). In no way can the volume recovery be accounted for as a purely passive process. This restoration of volume was particularly evident with ciliates transferred to 50% sea water. This aspect of volume regulation has been observed in few other protozoans (Kamada, 1935; Mast and Hopkins, 1941; Dunham and Stoner, 1967); there is no clear case of volume restoration in a marine ciliate (Kitching, 1967). Restoration of cell volume might be accomplished by maintenance of a rate of vacuolar output different from the rate of passive entry of water until volume was restored, after which time vacuolar output would be kept equal to passive entry in order to maintain constant cell volume. Redistribution of solutes, particularly Na^+ and Cl^-, after osmotic challenges must also play a role in regulation of cell volume.

The time required for the contractile vacuole to discharge a volume of fluid equal to the cell volume was 1.1 hour in 100% sea water. In fresh-water protozoans, turnover of cell water is much more rapid (4–45 min; Kitching, 1938). All marine ciliates have slower rates than fresh-water ciliates. Kitching (1938) has reported values of 2¾–4¾ hours for marine peritrichs. *M. avidus,* however, is smaller than the marine ciliates used in previous studies. With its larger surface/volume ratio, a higher rate of water entry per unit volume is expected.

SUMMARY

1. The euryhaline marine ciliate, *Miamiensis avidus,* was investigated for its ability to regulate solutes and water when exposed to different external salinities.

2. In 100% sea water-culture medium, *M. avidus* had the following inorganic ion concentrations (m*M*/kg cells): Na^+—87.9; K^+—73.7; Ca^{++}—3.7; Mg^{++}—28.5; Cl^-—60.8.

3. In 100% sea water-culture medium, $[Na^+]_i$, $[Cl^-]_i$, $[Mg^{++}]_i$ and $[Ca^{++}]_i$ were lower than the environmental values and $[K^+]_i$ was greater than $[K^+]_o$. $[Na^+]_i$ and $[Cl^-]_i$ changed with changes in external salinity, but were kept lower than $[Na^+]_o$ and $[Cl^-]_o$. $[K^+]_i$, $[Mg^{++}]_i$ and $[Ca^{++}]_i$ were maintained at fairly constant internal concentrations.

4. The contractile vacuole output was related to external osmolarity. Osmolarities greater than that of 100% sea water resulted in decreased vacuole output. In dilute sea water, output increased.

5. Cell volume determinations indicated a return toward the original volume after swelling or shrinking caused by transfer to media of different osmolarities.

6. The results suggest that *M. avidus* maintains itself hyperosmotic to the environment at all salinities. The contractile vacuole regulates cell volume by expelling water that enters passively.

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MINERAL REGENERATION BY SERPULID POLYCHAETE WORMS[1]

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Serpulid polychaete worms live in tubes which are constructed of an admixture of crystalline calcium carbonate and a mucopolysaccharide matrix material. Two glandular tissues participate in the secretion of the mineral and organic material of the tube. These are the calcium-secreting glands and the ventral shield epithelium (Swan, 1950; Hedley, 1956a, b; Vovelle, 1956). There are two calcium-secreting glands situated one on each side of the mid-ventral line embedded in the subepithelial connective tissue of the anterior peristomium under the fold of the collar. The ventral shield is a glandular epithelium surrounding the openings of the calcium-secreting glands on the surface of the ventral peristomium.

Several investigators have shown that the growth of the tubes of several species of serpulids can be very rapid (Hargitt, 1906; Harms, 1912; Dons, 1927; Fischer-Piette, 1937; Hill, 1967). However, no estimates have yet been published of the rate of $CaCO_3$ secretion by the calcium-secreting glands.

If carefully removed from their tubes and placed in sea water, many species of serpulids will within a few hours begin to secrete concretions of calcium carbonate from the calcium-secreting glands (Robertson and Pantin, 1938; Thomas, 1940; Swan, 1950; Vuillemin, 1954; Vovelle, 1956). These concretions have been called the mineral regenerate in the present investigation. The ventral shield epithelium probably does not contribute much to the formation of the mineral regenerate. Thus, the mineral regenerate can be used as a relatively precise indicator of the secretory activity of the calcium-secreting glands.

Two species of serpulids, *Hydroides brachyacantha* and *Eupomatus dianthus*, were used in the studies reported here, to examine (1) the ability of worms to secrete mineral regenerate at different salinities; (2) the rate of mineral regenerate production at different salinities; (3) the effect of the size (age) of the worm on the rate of mineral regenerate production; and (4) the relationship between the concentration of calcium in the tissues of the worm and the rate of mineral regenerate production.

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[1] This work represents part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Duke University. The experiments with *Hydroides brachyacantha* were conducted at the Instituto Oceanographico of the Universidade de São Paulo, at São Paulo and Ubatuba, Brazil. The experiments with *Eupomatus dianthus* were conducted at the Duke University Marine Laboratory at Beaufort, North Carolina. I wish to express my sincere thanks to Dr. Karl M. Wilbur, Department of Zoology, Duke University, and Dr. Edmundo Nonato, Instituto Oceanographico, Universidade de São Paulo, for their advice and encouragement during this study. This study was supported by Public Health Service Grants 5TI DE 92-05 and DE 02668 from the National Institutes of Health.

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Materials and Methods

Hydroides brachyacantha Rioja was collected from reef-like colonies of the sabellarid polychaete *Phragmatopoma lapidosa* Kinberg near the low-water mark of the exposed sandy beach, Praia do Tenorio, near Ubatuba in the state of São Paulo, Brazil. Small pieces of the reef containing serpulid tubes were removed and placed in 2–5 gallon aquaria filled with sea water. The aquaria were maintained at room temperature (18–23° C) and were aerated. The sea water in the aquaria was changed and dead animals removed every few days. Under these conditions serpulids could be kept alive for several weeks. However, all quantitative experimental studies with *H. brachyacantha* were conducted with worms that had been collected no more than one week previously.

Eupomatus dianthus (Verrill) was collected on shells and rocks lying just below the low water mark at Pivers Island, Beaufort, North Carolina. The worms in their tubes were placed in large wooden tanks with running sea water. The mortality of worms maintained in this way was very low and they could be kept in healthy condition indefinitely.

Studies of mineral regeneration

Recently collected serpulids were removed from their tubes in such a way as to minimize damage to their delicate abdominal and collar tissues. This was done in the following manner. The posterior end of the tube was carefully broken with a fine dissecting needle. The bristles of a fine camel's hair artist's brush were then inserted through the hole in the posterior end of the tube in order to push the worm forward in the tube. Then a little more of the posterior end of the tube was broken away and the bristles inserted again. This was continued until only a short length of the anterior part of the tube remained and the worm was forced to extend its branchial crown from the anterior end of the tube. Finally, the worm was pushed back into the tube and out the broken posterior end with the artist's brush.

When removed from their tubes, the worms usually released eggs and sperm into the water. After this had stopped, uninjured worms were placed in petri dishes containing 50–100 ml full strength sea water (salinity 33–34‰), dilute sea water, or hypercalcium sea water. Dilute sea water was prepared by mixing distilled water with full strength sea water. Hypercalcium sea water was prepared by adding reagent grade $CaCl_2$ to standard sea water. The salinity of all sea-water samples was determined by the hydrometer method.

From 10 to 20 worms, all about the same size, were placed in each petri dish. The worms were allowed to regenerate mineral for 24 hours. At the end of 24 hours, the worms were examined under a dissection microscope and the relative amount of mineral regenerate which had accumulated under the collar of each worm was recorded. The mineral regenerate was carefully removed with a pair of fine forceps, rinsed in dilute NaOH solution (pH 7.5–8.5), and placed in 12-ml conical Pyrex centrifuge tubes. The mineral regenerate material from the 10–20 worms of the same size in each petri dish was pooled for each calcium determination. The worms were then removed from the sea water, blotted on absorbent ash-free filter paper, and weighed on an analytical balance to the nearest 0.1 mg. The pooled worms were then placed in 16 × 125 mm high temperature Pyrex ignition tubes

(Corning #9880) and ashed in a muffle furnace at 550–600° C for 5–8 hours. Samples of the sea water from the petri dishes were also collected for determination of calcium.

Calcium determinations

Both the mineral regenerate and the ash were dissolved in 1 ml of 1 N HCl and then brought to neutral pH by the addition of an equivalent amount of 1 N NaOH. The samples were then buffered at pH 5.6 with 1 ml of 0.2 M acetate buffer. Calcium was determined by the chloranilate method of Ferro and Ham (1957). The calcium concentration in duplicate 0.5–1.0-ml aliquots of the sea water samples was determined by the same technique without prior addition of HCl, NaOH or acetate buffer.

Mineralogy of tubes and mineral regenerate

Clean serpulid tubes free of adhering foreign matter were selected for examination by x-ray diffraction techniques. Mineral regenerate material was collected as described above for x-ray diffraction analysis. The mineral samples were rinsed

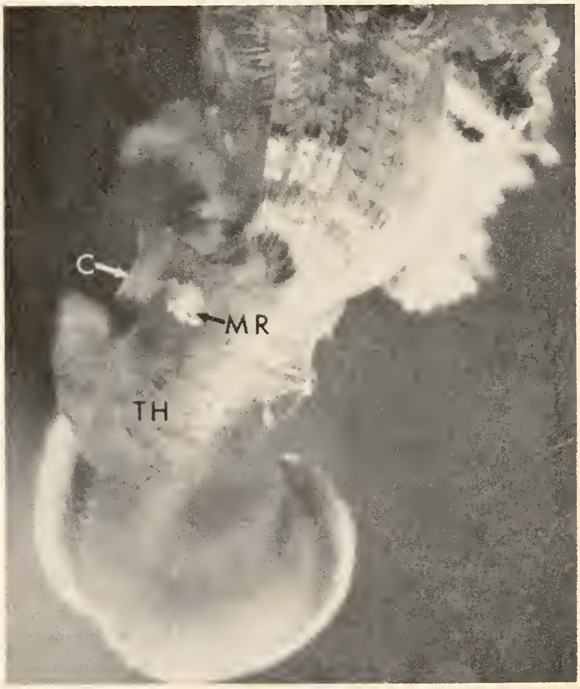

FIGURE 1. *E. dianthus* removed from its tube and allowed to regenerate mineral for about 4 hours. Side view. A small mineral concretion, the mineral regenerate (MR), lies under the fold of the collar and directly over the opening of the left calcium-secreting gland. Another concretion, not seen in this photograph, lies over the other calcium-secreting gland on the right side of the ventral peristomium. 20 ×. C, collar; MR, mineral regenerate; TH, thoracic membrane.

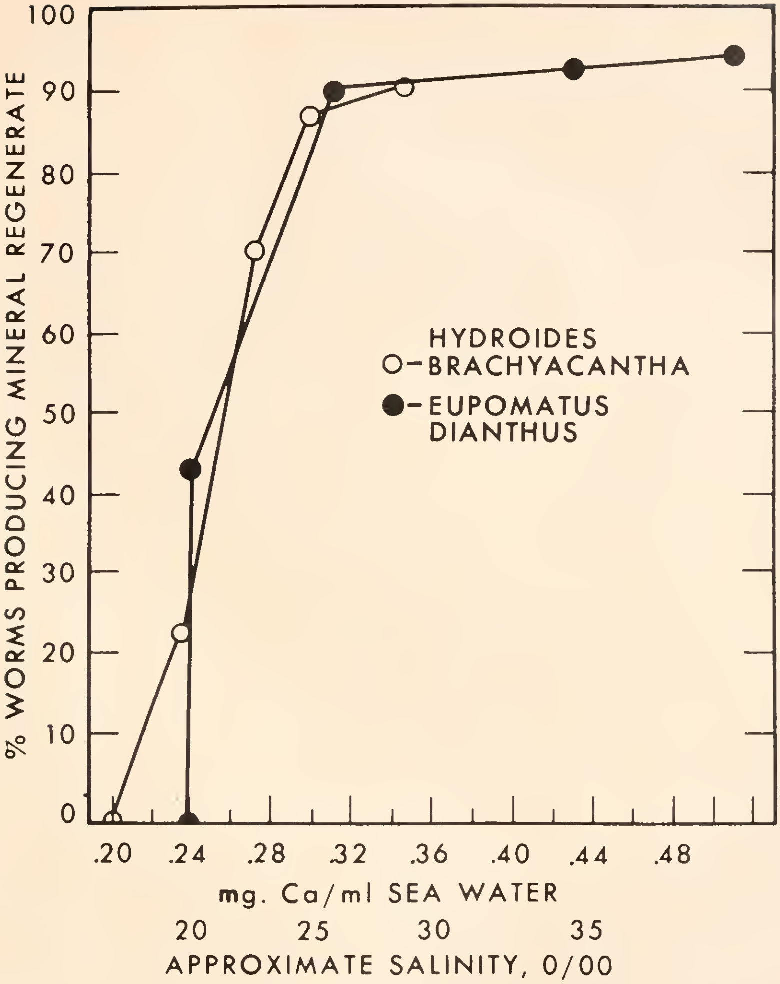

FIGURE 2. The effect of salinity on the ability of *H. brachyacantha* and *E. dianthus* to produce mineral regenerate. Regeneration ability is expressed as the percentage of worms which produced mineral regenerate within 24 hours after being removed from their tubes. In this and subsequent graphs, hypercalcium sea water (0.49 mg Ca/ml) prepared by adding $CaCl_2$ to standard sea water, has approximately the same salinity as standard sea water (34‰, 0.43 mg Ca/ml).

in several changes of distilled water and allowed to dry at room temperature. They were then ground to a fine powder with an agate mortar and pestle and placed in 0.3-mm (o.d.) lithium-borate glass capillary tubes (Lindemann Co., West Germany). X-ray diffraction pictures were taken with a Norelco 11.46 cm Debye-Scherrer x-ray diffraction camera. The x-ray source was a Norelco x-ray machine with Cu Kα radiation at 35 KV and 20 ma. Exposure time was 2–5 hours.

The presence of substituted magnesium in the calcite lattice (high Mg calcite) was estimated by the downward displacement of the 104 "d" line in the diffraction pattern as described by Chave (1952). However, no attempt was made to quantitatively estimate the amount of Mg in the calcite or the ratios of calcite to aragonite in the mineral samples.

RESULTS

Formation of the mineral regenerate

When serpulids were carefully removed from their tubes and placed in sea water, small concretions of mineral, the mineral regenerate, began to form within a few hours. The mineral regenerate first appeared under the fold of the collar directly over the openings of the two calcium-secreting glands (Fig. 1). With time, the mineral regenerate increased in size, first spreading laterally to fill the area under the fold of the collar and later extending posteriorly in the form of one or two thin sheets. At the end of 24 hours the mineral regenerate often extended to the poste-

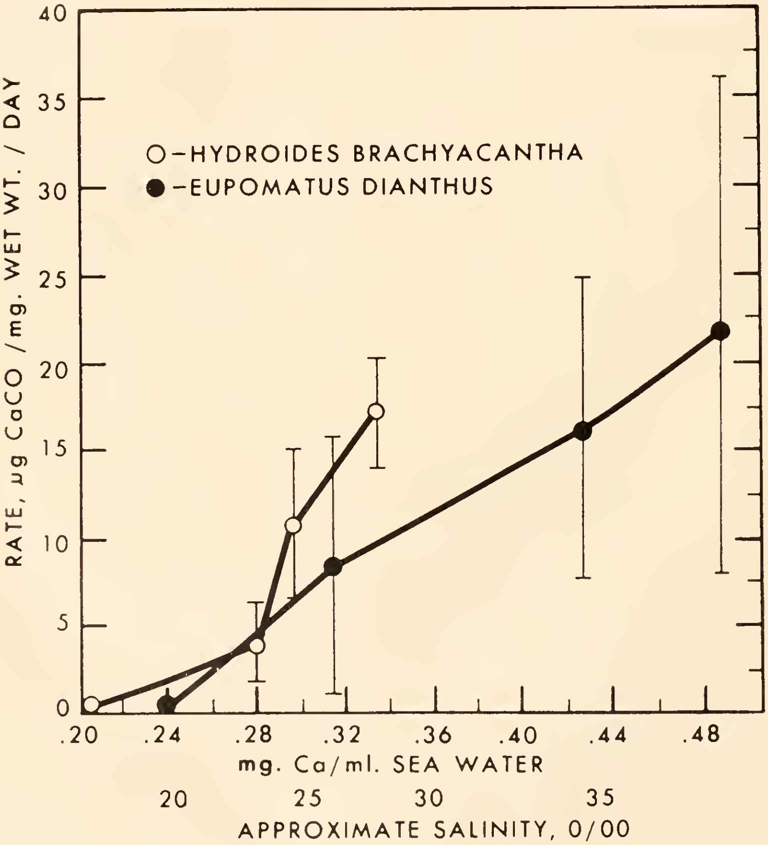

FIGURE 3. The effect of salinity on the relative rate of mineral regenerate production by *H. brachyacantha* and *E. dianthus*. Each point is the mean of 7 to 16 determinations on pooled samples of 10 to 20 animals each. The ranges indicate the sample standard deviations.

TABLE I

Statistical analysis of the relationship between the relative rate of mineral regenerate production by H. brachyacantha *and* E. dianthus. *"P" values are based on "student T test" for significance between means (Snedecor, 1956)*

Species	mg Ca/ml sea water (salinity)	Sample size	Mean rate μg Ca/mg wet wt/day	Sample standard deviation	*P* value
Hydroides brachyacantha	0.270 (23‰)	7	1.70	0.75	<0.01
	0.285 (25‰)	9	4.33	1.63	<0.01
	0.330 (28‰)	16	6.86	1.29	
Eupomatus dianthus	0.318 (26‰)	7	3.30	3.15	0.05
	0.430 (34‰)	16	6.30	3.17	0.25
	0.490 (34‰)	6	8.70	5.44	

rior border of the thorax. However, the only points of attachment between the worms and the mineral regenerate were at the openings of the calcium-secreting glands.

Minerology of the tubes and mineral regenerate

The tubes of *H. brachyacantha* and *E. dianthus* contained large amounts of two polymorphs of calcium carbonate, high magnesium calcite and aragonite. The tubes sometimes also contained a small amount of α-quartz which probably represented inclusions of sand grains in the mineral material of the tube. Most samples of mineral regenerate material contained only aragonite. However, a few also contained traces of high magnesium calcite. Low magnesium calcite and vaterite were not detected in the tubes or mineral regenerate material of either species.

Mineral regenerate production and salinity

The ability of serpulids which have been removed from their tubes to produce mineral regenerate is influenced by several factors. One is salinity. The ability of two species of serpulids, *Hydroides brachyacantha* and *Eupomatus dianthus,* to produce mineral regenerate at different salinities is summarized in Figure 2. Mineral regeneration ability was expressed as the percentage of individuals which produced mineral regenerate within 24 hours after being removed from their tubes. A total of 540 *H. brachyacantha* and 670 *E. dianthus* was used for these determinations. Both species of serpulids responded similarly to changes in the salinity with respect to their ability to produce mineral regenerate. The relative success of both species in producing mineral regenerate rose steeply between salinities of about 20‰ and 25‰. At salinities above about 25‰ (corresponding to a sea water calcium concentration of about 0.30 mg Ca/ml), 88% or more of *H. brachyacantha* and *E. dianthus* produced mineral regenerate. Ninety-five per cent of *E. dianthus* in hypercalcium sea water (0.49 mg Ca/ml) produced mineral regenerate within 24 hours.

The rate of mineral regenerate production was affected by the salinity and possibly also by the calcium concentration of the sea water. The relationship between the rate of mineral regenerate production by *H. brachyacantha* and *E. dianthus* and the salinity, expressed as the calcium concentration of sea water, is shown in Figure 3. In both species, the rate of mineral regenerate production per unit wet weight of worms rose with increasing salinity. There was a significant increase in the rate of mineral regenerate production by *E. dianthus* between salinities of 26‰ (0.32 mg Ca/ml sea water) and 34‰ (0.43 mg Ca/ml sea water) ($P = 0.05$), but not between 34‰ normal sea water and hypercalcium sea water (0.49 mg Ca/ml sea water) ($P = 0.25$). In the range of salinities in which mineral regenerate production by *H. brachyacantha* was observed, the observed increases in the rate of mineral regenerate production with increasing salinity were significant ($P < 0.01$). Statistical data are summarized in Table I.

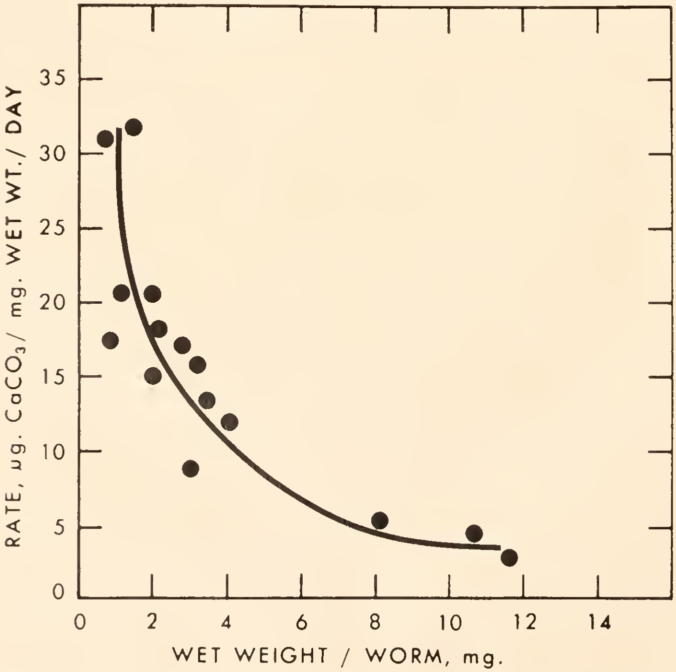

FIGURE 4. The relative rate of mineral regenerate production as a function of size in *E. dianthus* at a salinity of 34‰ (0.43 mg Ca/ml sea water). Each point represents a pooled sample of 10 to 20 individuals of approximately the same size. The average curve was fitted by inspection and approximates an exponential function.

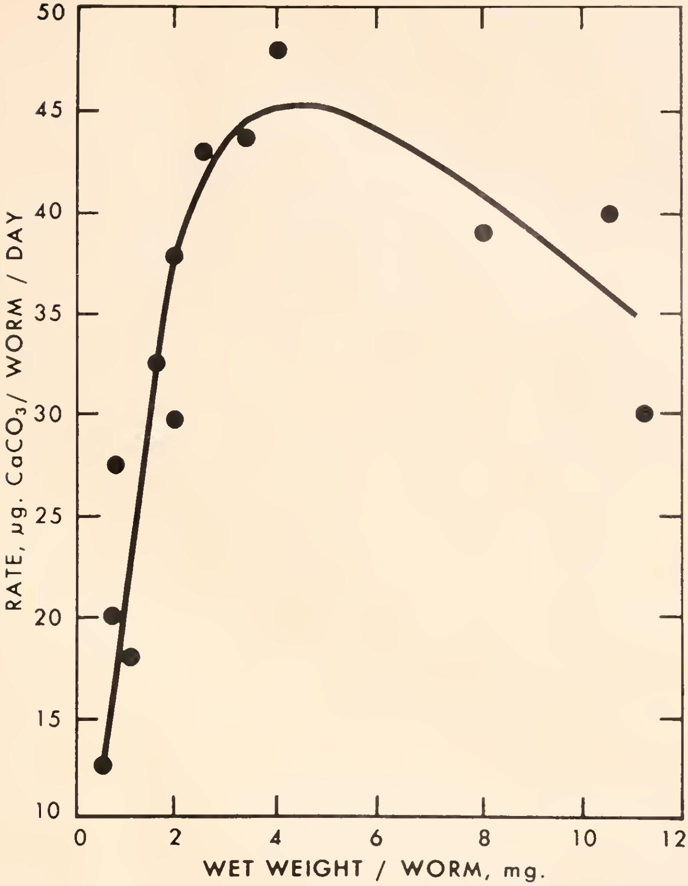

FIGURE 5. The effect of size of *E. dianthus* on the rate of mineral regenerate production per worm at a salinity of 34‰ (0.43 mg Ca/ml sea water). Each point represents a pooled sample of 10 to 20 individuals of approximately the same size. The average curve was fitted by inspection.

At each salinity there was a large variation in the rate of mineral regenerate production by both species. This variability was expressed as the sample standard deviation (Snedecor, 1956) in Table I. The rate of mineral regenerate production by *H. brachyacantha* was less variable than that by *E. dianthus* at each salinity.

Size and the rate of mineral regenerate production

Much of the observed variability in the rate of mineral regenerate production at different salinities can be attributed to differences in the rate of mineral regenerate production by worms of different sizes. Figure 4 shows the effect of size on the relative rate of mineral regenerate production by *E. dianthus* in normal sea water. The rate decreased approximately exponentially with increasing size of individuals.

Thus, the smallest worms with an average wet weight of less than 1 mg produced a maximum of 30–33 μg $CaCO_3$/mg wet wt/day, while the largest worms examined averaging 10–12 mg wet weight, produced mineral regenerate at about one tenth this rate.

However, the rate of mineral regenerate production per worm (Fig. 5) increased sharply with increasing size and passed through a maximum at 4 mg. In worms larger than 4 mg, the rate of mineral regenerate production per worm decreased with increasing size of individuals. Worms averaging 4 mg wet weight produced nearly 50 μg $CaCO_3$/day, equivalent to about 1.25% of their body weight.

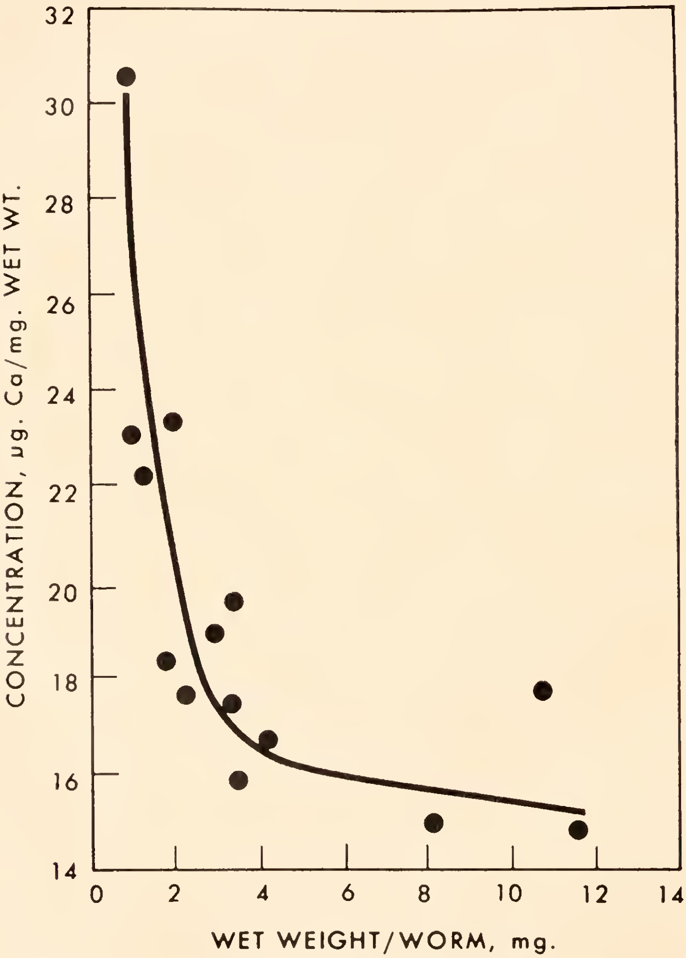

FIGURE 6. The relationship between the concentration of tissue calcium of *E. dianthus* and size. Salinity, 34‰ (0.43 mg Ca/ml sea water). Each point represents a pooled sample of 10 to 20 individuals of approximately the same size. The average curve was fitted by inspection and approximates an exponential function.

Tissue calcium in Eupomatus dianthus

There was an inverse exponential relationship between the concentration of tissue calcium and the size of the worms (Fig. 6). The concentration of tissue calcium of whole worms fell from about 3.0 μg Ca/mg wet wt tissue (75 mM Ca/kg) in the smallest worms to about 1.5 μg Ca/mg wet wt tissue (37.5 mM Ca/kg) in the largest. These concentrations of tissue calcium were very high as compared with the concentration of calcium in the tissues of most other marine invertebrates. The concentration of tissue calcium in other marine invertebrates is rarely higher than about 17 mM/kg (Prosser, 1961).

Tissue calcium and mineral regenerate production

The rate of mineral regenerate production by *E. dianthus* was related not only to the salinity of the sea water and the relative size of the worms, but also to the concentration of tissue calcium (Fig. 7). There was a linear relationship between

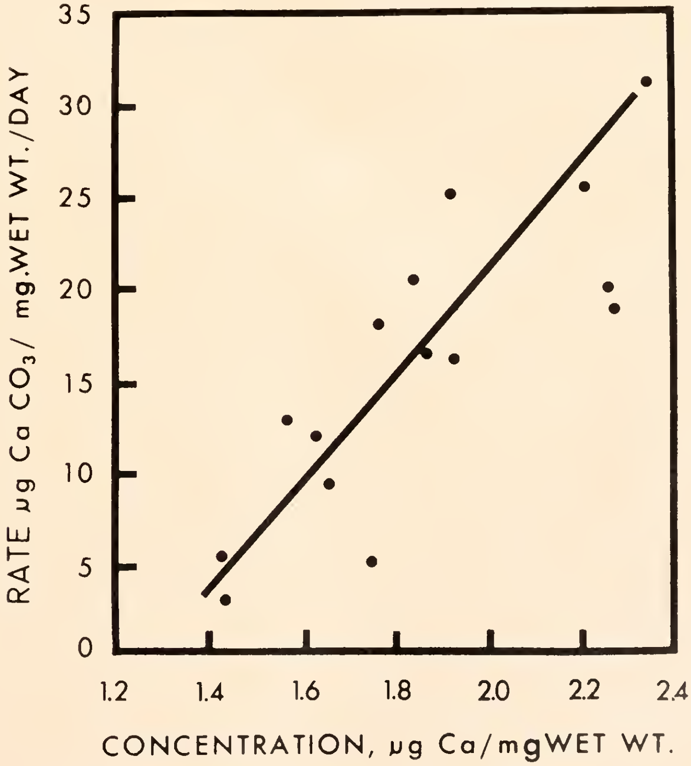

FIGURE 7. The relationship between the tissue calcium concentration and the relative rate of mineral regenerate production of *E. dianthus* at a salinity of 34‰ (0.43 mg Ca/ml sea water). Each point represents the results of a determination of both parameters on a single pooled sample of 10 to 20 worms of approximately the same size. The average curve was fitted by inspection.

tissue calcium concentration and the relative rate of mineral regenerate production. The coefficient of correlation (Snedecor, 1956) for the relationship between the relative rate of mineral production and the tissue calcium concentration was high (0.86) in worms allowed to regenerate mineral in normal sea water (0.43 mg Ca/ml sea water) (Table II). At lower and higher levels of environmental calcium, the coefficient of correlation was lower.

The ratio of the rate of mineral regenerate production per unit wet weight of worms to the concentration of tissue calcium varied between 3.34 and 4.33 (Table II) over the range of environmental calcium concentrations studied. That is, the worms secreted three to four times the amount of tissue calcium per day.

Table II

The relationship between the tissue calcium concentration and the relative rate of mineral regenerate production by E. dianthus *at different salinities and environmental calcium concentrations*

Calcium concentration of the sea water, mg Ca/ml	Ratio of rate of mineral production to tissue calcium concentration	Coefficient of correlation, R	Number of samples used. 10–20 worms per sample
0.318	4.33	0.64	7
0.427	3.39	0.86	11
0.490	3.34	0.68	8

Discussion

The x-ray diffraction data indicate that the mineral regenerate represents only a part of the material found in the normal tube. Muzii (personal communication) found that the tube of *E. dianthus* was composed of three distinct mineral layers, an outer aragonitic layer and two inner calcitic layers. The observations of mineral regenerate production strongly suggest that the mineral regenerate material is secreted by the calcium-secreting glands alone. This conclusion is supported by the observation that the lumen of the calcium-secreting gland sometimes contained aragonite (Neff, 1967).

Hedley (1956a, b) and Vovelle (1956) found that the columnar mucocytes of the ventral shield epithelium contained a high concentration of calcium and suggested that they may secrete part of the mineral destined for the tube. The ventral shield may secrete the calcitic layers of the tubes of *E. dianthus* and *H. brachyacantha.* However, it apparently does not participate in the formation of the mineral regenerate. Therefore, mineral regeneration may be used as an indicator of the secretory activity of the calcium-secreting glands.

The production of mineral regenerate by serpulids which have been removed from their tubes may represent an attempt by the worm to produce a new tube. However, the amount of mineral regenerate produced by worms during 24 hours varied tremendously from one worm to another and not all worms produced mineral regenerate within this length of time. Vovelle (1956) noted that, in the serpulid *Pomatoceros triqueter,* the time before the first appearance of mineral regenerate varied from less than 2 to more than 24 hours. He suggested that the calcium-secreting glands underwent cyclical changes in secretory activity and the ability of the worms to produce mineral regenerate depended on the activity stage of the calcium-secreting glands at the time the worms were removed from their tubes. Furthermore, he observed that many *P. triqueter* stopped producing mineral regenerate after about 48 hours and concluded that the calcium-secreting glands had become exhausted. However, Faouzi (1931) reported that some specimens of *P. triqueter* continued to produce mineral regenerate for as long as 40 days.

Robertson and Pantin (1938) found that *P. triqueter* was unable to produce mineral regenerate in artificial sea water containing less than 50% of the normal concentration of calcium. More extensive experiments on the relationship between sea water calcium concentration and the rate of mineral regenerate production have been presented in the present investigation.

The present observations that both species of serpulids failed to produce mineral regenerate below a salinity of about 20‰ and that above this salinity the rate of mineral regenerate production increased with increasing salinity and sea water calcium concentration lend support to the conclusion of Robertson and Pantin (1938) that serpulids utilize dissolved calcium for the construction of their tubes. There are two ways in which dissolved calcium could be utilized for the construction of the calcified tube. Calcium could be precipitated directly from sea water onto an organic matrix material after the latter is secreted by the worm. On the other hand, calcium could be taken up from the sea water by various tissues of the worm and transported to the calcium-secreting glands and ventral shield epithelium where it might be mixed with the organic matrix material before being secreted to form the tube. All the available evidence indicates that the latter scheme is the more likely. Swan (1950), using Sr^{85}, showed that at least part of the calcium destined for incorporation in the tube passed through the worm. Hedley (1956a, b) and Vovelle (1956) found high concentrations of calcium in the calcium-secreting glands and ventral shield epithelium, strongly indicating that these tissues secrete calcium as well as matrix material.

Recent studies of the ultrastructure of the calcium-secreting glands of *Pomatoceros caeruleus* (Neff, 1966, 1967) have shown that the primary secretory product of these glands in this species has the form of highly organized granules of crystalline calcite. In *Eupomatus dianthus,* microcrystals of aragonite have been observed in the upper part of the lumen of the calcium-secreting glands (Neff, 1967).

Potential sites for the uptake of calcium from the sea water have been identified in the epithelia of the anterior surface of the collar and base of the branchial crown of *P. caeruleus* (Neff, 1967, 1968). Swan (1950) and Vovelle (1956) described similar calcium-rich epithelia in the lining of the anterior gut of *Mercierella enigmatica* and *Pomatoceros triqueter* and suggested that they may function in the uptake and storage of calcium from the gut.

The non-linear relationship between salinity and both the ability of worms to produce mineral regenerate and the rate at which it is formed would be hard to explain in terms of precipitation of $CaCO_3$ directly from sea water onto the organic matrix. However, the possibility that some $CaCO_3$ is precipitated directly from the sea water onto the mineral-matrix material secreted by the worm cannot be completely discounted.

The highest rate of mineral regenerate production was observed in *E. dianthus* weighing about 4 mg. Worms of this size produced approximately 50 μg of aragonite per day. Hedley (1956b) described the morphology of the calcium-secreting glands of a closely related serpulid *Hydroides norvegica.* He indicated that the calcium-secreting glands were simple tubular organs with an average length of 200–250 μ and a diameter of 40–50 μ in worms probably somewhat larger than 4 mg (worms 15 mm long). Recent ultrastructural studies by the author (Neff, 1967) have shown that the calcium-secreting glands of *E. dianthus* have a similar morphology and roughly similar dimensions. The central oval gland duct has an average diameter of about 10 μ. Thus the total cell volume of each calcium-secreting gland is about $9.5 \times 10^5 \mu^3$. Each gland secretes approximately 1 μg aragonite/hour equivalent to a volume of $3.3 \times 10^5 \mu^3$. Thus, each gland secretes an amount of mineral equivalent to about $\frac{1}{3}$ its cell volume per hour.

It has been shown in the present investigation that the concentration of calcium in the tissues of *E. dianthus* was very high as compared with the concentration of calcium in the tissues of most other marine invertebrates. However, several species of marine molluscs (McCance and Shackleton, 1937) and the holothurian *Caudina* (Koizumi, 1935) have tissues with a calcium concentration as high or even higher than that in serpulids. Much of the calcium was concentrated in the tissues in the form of small mineral concretions or spicules (McCance and Masters, 1937; Koizumi, 1935). In serpulids also, much of the tissue calcium was apparently associated with discrete mineral concretions in various tissues. Hedley (1956a, 1958) observed that the secretory cells of the lumina of the calcium-secreting glands as well as the mucocytes of the ventral shield epithelium and the dorsal surface of the last few abdominal segments of *Pomatoceros triqueter* contained high concentrations of calcium. Swan (1950) and Vovelle (1956) identified calcium-storage tissues containing calcium-rich granules in the anterior intestinal epithelium, the nephridia, and the chloragosomes of *Mercierella enigmatica* and *Pomatoceros triqueter.* Granules of crystalline calcite have been identified in the secretory cells and ducts of the calcium-secreting glands of *Pomatoceros caeruleus* (Neff, 1966, 1967) and intracellular hydroxyapatite crystals have been found in the calcium-uptake tissues on the anterior surface of the collar and base of the branchial crown of *Pomatoceros caeruleus* (Neff, 1967, 1968). All these calcium-rich tissues in serpulids probably play a role in some phase of mineral secretion. Although allometric data concerning the relative rates of growth of different tissues in serpulids are completely lacking, the observed exponential decrease in the total tissue calcium concentration with increase in size of worms and the close relationship between the rate of mineral regenerate production and the concentration of tissue calcium suggest that the mass of these calcium-rich tissues does not increase as rapidly as the mass of the whole worm.

SUMMARY

1. The tubes of *H. brachyacantha* and *E. dianthus* contained two polymorphs of calcium carbonate, high magnesium calcite and aragonite, whereas the mineral regenerate produced by both species contained only aragonite. The site of initial appearance of the mineral regenerate over the openings of the calcium-secreting glands and the presence of aragonite in the duct of the calcium-secreting gland of *E. dianthus* indicate that the aragonite of the mineral regenerate and probably also of the tube is secreted by the calcium-secreting glands. The high magnesium calcite

fraction of the tube is probably secreted by the ventral shield epithelium.

2. At all salinities in which worms were able to produce mineral regenerate the rate of mineral regenerate production was extremely variable.

3. Both species of serpulids failed to produce mineral regenerate below a salinity of about 20‰. Above this salinity the rate of mineral regenerate production increased with increasing salinity and environmental calcium concentration. However, there was not a significant increase in the rate of mineral production by *E. dianthus* between normal sea water (34‰, 0.430 mg Ca/ml) and hypercalcium sea water (0.490 mg Ca/ml).

4. *E. dianthus,* weighing about 4 mg, secreted up to 50 μg of $CaCO_3$ per day. Thus worms of this size secreted an amount of aragonite equivalent to about ⅓ of the cell volume of the calcium-secreting glands per hour.

5. There was an inverse exponential relationship between the size of worms and both the relative rate of mineral regenerate production and the concentration of calcium in the tissues of the worms, strongly suggesting that the mass of the tissues involved in mineral production did not increase in proportion to the increase in mass of the worm.

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NATURAL AND SYNTHETIC MATERIALS WITH INSECT HORMONE ACTIVITY. 2. JUVENILE HORMONE ACTIVITY OF SOME DERIVATIVES OF FARNESENIC ACID

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A number of substances with juvenile hormone activity have been isolated from natural sources (Bowers *et al.*, 1966; Černý *et al.*, 1967; Röller *et al.*, 1967; Schmialek, 1961) or prepared synthetically (Ayyar and Rao, 1967; Dahm *et al.*, 1967; Mori and Matsuji, 1967; Romaňuk *et al.*, 1967; Schmialek, 1963; Schneiderman *et al.*, 1965; Sláma *et al.*, 1968) during the past few years. Most of these substances are derived from terpenes of farnesane or bisabolane types. Law *et al.* (1966) discovered that the reaction between hydrogen chloride and an alcoholic solution of farnesenic acid produced a mixture of extremely active juvenile hormone analogues. The authors observed that the activity of the reaction products was dependent on the alcohol used, ethanol giving maximum activity.

It was later found (Romaňuk *et al.*, 1967) that for *Pyrrhocoris apterus* the most active components of this mixture were esters of farnesenic acid (I) in which the two double bonds were saturated by hydrogen chloride (VII-IX). Subsequently, in our search for new juvenile hormone analogues we have synthesized a number of farnesenic acid derivatives with pronounced changes in biological activity. In the present communication we give an account of the juvenile hormone activity of some selected derivatives when assayed on four species of insects.

MATERIALS AND METHODS

Juvenile hormone activity was determined by topical assays on *Pyrrhocoris apterus* and *Dysdercus cingulatus* (Hemiptera, Pyrrhocoridae), on *Graphosoma italicum* (Hemiptera, Pentatomidae) and by injection assays on pupae of *Tenebrio molitor* (Coleoptera, Tenebrionidae). In the topical tests we applied the substances in 1 μl acetone to the abdominal tergites of freshly molted last instar larvae. In the *Tenebrio* assay, we injected the substances in 1 μl olive oil into freshly molted pupae. The activity was determined by the degree of inhibition of metamorphosis and was expressed in juvenile hormone units (Sláma, 1968). One unit indicates the amount of substance, in micrograms per specimen, which caused half-larval, half-adult intermediates (Hemiptera) or half-pupal, half-adult intermediates (*Tenebrio*). In Hemiptera the whole range of activity from zero (normal adults) to the maximum (supernumerary larvae) was realized with a ten-fold change in concentration. Thus, when one unit is determined as 0.05, it indicates that the compound shows a trace of activity at 0.01, medium activity at 0.05, and maximum activity at 0.1 μg per specimen. In *Tenebrio* the activity range from zero to maximum extended over 100- to 1000-fold

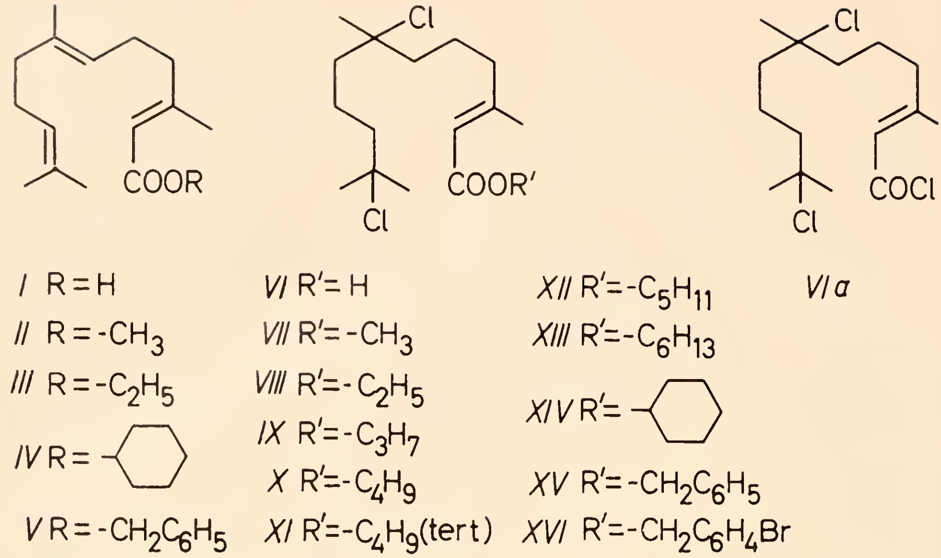

Figure 1.

changes in concentration. Serial dilution were used to determine the range of activity of each compound; then, assays within this concentration range permitted a more accurate determination of activity. If the compounds did not show any activity below 100 μg in topical assays or 1000 μg in injection assays, no further tests were performed.

The compounds (I)-(III) were prepared by conventional methods; (IV) and (V) were synthesized by alkylation of silver salts of farnesenic acid with cyclohexyl and benzyl iodide respectively. For the synthesis of esters (VII)-(XVI) we used crystalline *trans*-dihydrodichlorofarnesenic acid (VI) (*trans*-3,7,11-trimethyl-7,11-dichloro-2-dodecenic acid, m.p. 93–94° (Romaňuk and Šorm, 1968). The acid was prepared by bubbling gaseous hydrogen chloride through an acetic acid solution of farnesenic acid. It was then treated with thionyl chloride to produce the acid chloride (VIa) which, in turn, was reacted with various alcohols to give the corresponding *trans* dichloro-esters.

Farnesol and farnesyl methyl ether were commercial samples which were checked for purity by gas chromatography. Methyl 10,11-epoxyfarnesoate was made in a usual way by action of perphthalic acid on methyl farnesoate. The preparation of p-(dimethyl-hexyl) benzoic acid derivatives has already been described (Sláma *et al.*, 1968). The synthetic *d,l*-juvenile hormone was a gift from Dr. H. Röller. "Juvabione" (methyl todomatuate) was the natural product isolated from balsam fir wood (Černý *et al.*, 1967).

Results and Discussion

The results are summarized in Table I. Farnesenic acid (I) had generally low activity, one unit being in all cases much greater than 100 μg. Its methyl and ethyl esters (II), (III) had low activity on the hemipterans (1 unit = 10–50 μg) and substantial activity for *Tenebrio* (1 unit = 5–10 μg). The cyclohexyl and benzyl farnesoates (IV), (V) again had lower activity. Addition of hydrogen chloride to the double bonds of farnesenic acid and its esters (VI)-(XVI) was followed by drastic changes in the juvenile hormone activity for the hemipterans but not for *Tenebrio*. Thus, the dihydrodichlorofarnesenic acid (VI) was about 100 times more active in the hemipterans than the original farnesenic acid (I); the activity of the dihydrodichloro compounds increased from the acid (VI) to the methyl ester (VII) and ethyl ester (VIII) where it attained its maximum. It decreased again with increasing number of carbon atoms in the ester radical from the propyl ester (IX) to the cyclohexyl ester (X) to (XIV). The aralkyl esters (XV), (XVI) were again slightly more active on *Pyrrhocoris* and *Dysdercus*. These data support the earlier observations of Law *et al.* (1966) who found that the reaction between hydrogen chloride and farnesenic acid produced the most active juvenile hormone materials in the presence of ethanol.

The most active compound we tested for juvenile hormone activity was the ethyl ester of *trans*-dihydrodichlorofarnesenic acid (VIII) which, when compared

TABLE I

Juvenile hormone activity units (indicated by an amount of the substance in μg per specimen which causes half-larval or half-pupal adultoids)

	Topical assays on larvae of			Injections into pupae of *Tenebrio*
	Pyrrhocoris	*Dysdercus*	*Graphosoma*	
(I) Farnesenic acid (FA)	>100	>100	>100	>100
(II) FA methyl ester	10–50	50	50	5–10
(III) FA ethyl ester	50	30	50	5
(IV) FA cyclohexyl ester	50	100	100	100
(V) FA benzyl ester	100	100	100	1000
(VI) Dihydrodichloro farnesenic acid (DFA)	1	1	>100	>1000
(VII) DFA methyl ester	0.0008	0.01	30	1000
(VIII) DFA ethyl ester	0.0005	0.003	1	100
(IX) DFA propyl ester	0.005	0.008	100	1000
(X) DFA n-butyl ester	0.5	0.5	>100	>1000
(XI) DFA tert. butyl ester	0.1	0.05	>100	>1000
(XII) DFA amyl ester	3	0.5	>100	>1000
(XIII) DFA hexyl ester	4	1	>100	>1000
(XIV) DFA cyclohexyl ester	3	5	>100	>1000
(XV) DFA benzyl ester	0.4	0.2	>100	>1000
(XVI) DFA p-bromobenzyl ester	0.09	0.4	>100	>1000
Farnesol	>100	>100	>100	100
Farnesyl methyl ether	30	10	100	10
10,11-epoxymethylfarnesoate	3	1	10	10
d,l-juvenile hormone	0.5	0.1	1	0.5
"Juvabione" (methyl todomatuate)	3	1	>100	>1000
p-(1,5-dimethyl-hexyl) benzoic acid methyl ester	0.5	0.1	>100	>1000
p-(1,5-dimethyl-1,5-dichlorohexyl) benzoic acid methyl ester	0.3	0.04	>100	>1000

with ethylfarnesoate (III), is 10^5 times more active on *Pyrrhocoris,* 10^4 times more active on *Dysdercus,* and 50 times more active on *Graphosoma.* As little as 0.1 nanogram of the compound (VIII) applied to *Pyrrhocoris* larvae produced adultoids which were unable to survive and reproduce. Thus, the minimum effective concentration appears to be 2.5 μg per kilogram live weight, or 2.5 mg per metric ton of insects representing approximately 25 million larvae. Experiments in which the larvae were reared on a filter paper impregnated with substance (VIII) revealed that a dose of 1 μg per square meter of filter paper was still effective. In practical terms, this shows that 10 mg per hectare could in principle prevent development of *Pyrrhocoris.*

Unlike the hemipteran insects, the pupae of *Tenebrio* were quite insensitive to the esters of dihydrodichlorofarnesenic acid. Their activity, in fact, was significantly decreased when compared with the esters of farnesenic acid. Thus, the same chemical changes of the molecular structure which lead to 10^5-fold increase of juvenile hormone activity in one species (*Pyrrhocoris*) may be followed by considerable loss of activity when assayed on another species. According to the present evidence, differences in insect sensitivity to these compounds occur, not only between hemipterans and *Tenebrio,* but also among the hemipterans themselves. As seen in Table I, the larvae of *Graphosoma* (Pentatomidae) were about equally sensitive to the unsubstituted esters of farnesenic acid as were the larvae of *Pyrrhocoris* and *Dysdercus* (Pyrrhocoridae), but much less sensitive to the dihydrodichloro compounds. It will also be observed that "juvabione" and *p*-(dimethyl-hexyl) benzoic acid esters (Sláma *et al.,* 1968) act only on the pyrrhocorid bugs and not on the pentatomid bug. Due to the observed selective effects on different species we expect that some of the analogues with relatively low juvenile activity on hemipterans or *Tenebrio* may later appear to be quite active on certain other insects.

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SUMMARY

The juvenile hormone activity of both farnesenic acid esters and their dihydrodichloro derivatives increased from the methyl to the ethyl ester, then decreased again. In some hemipteran insects enormous increases in activity were obtained after addition of two hydrogen chloride molecules to the two double bonds of farnesenic acid esters. The same change in chemical structure considerably decreased the activity on *Tenebrio* pupae. On *Pyrrhocoris apterus,* the highest activity (at the 0.1 nanogram level) was obtained with the ethyl ester of *trans*-3,7,11-trimethyl-7,11-dichloro-2-dodecenic acid.

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THE MORPHOLOGY AND LIFE-HISTORY OF *NEOPECHONA PYRIFORME* (LINTON, 1900) N. GEN., N. COMB. (TREMATODA: LEPOCREADIIDAE) [1]

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Linton (1900) described *Distomum pyriforme* n. sp., from the pyloric ceca and intestine of the rudderfish, *Palinurichthys perciformis,* taken at Woods Hole, Massachusetts, on four occasions in August, 1898. The description states, p. 292, "Body very slightly compressed, of various shapes, but usually elliptical or pyriform in outline, armed with low, flat, rounded, scale-like spines. Neck in some slightly extended; in others the oral sucker was retracted (Fig. 56)." The latter feature appears to be characteristic since Linton noted, p. 293, "A large portion of the preserved specimens have the anterior end of the body inverted." His Figure 57 was made from a longitudinal, frontal section showing the inverted anterior end of a worm with a tubular canal from the retracted oral sucker to the surface of the body. The species was illustrated with Figures 52–59. The type material consists of more than 100 specimens, mounted on eight slides, deposited in the Helminthological Collection of the U. S. National Museum under the number 6516. All of the worms are juvenile, with immature gonads and diffuse pigment, from disintegrating ocelli, in the lateral pharyngeal areas. The measurements of specimens were made on young, half-grown individuals and have little value. The account, however, contains much significant information, especially the report on living specimens, some of which were mature, since there are observations on the spinose cirrus, the relatively large seminal vesicle and prostate, voluminous vitellaria, and the size of eggs. Figures 55 and 56 are particularly interesting; they demonstrate the confused and inadequate status of the specific description. In Figure 55, the ceca terminate at the anterior border of the excretory vesicle, midway between the acetabulum and the posterior end of the body, while the excretory vesicle is represented as saccate, extending forward only to the caudal ends of the ceca. It is probable that the caudal portions of the ceca and the anterior extension of the excretory vesicle were not observed since in Figure 56, the same structures are represented very differently; the ceca extend to the posterior end of the body while the excretory vesicle extends forward to the level of the acetabulum and contains a row of concretions. The text, p. 292 states, "Intestinal branches conspicuous, straight, reaching the posterior end of the body." On p. 293 there is the statement, "Spherical bodies with a concentric structure were seen lying in the excretory vesicle. These masses were not of uniform size; the largest measured 0.010 mm in diameter. They appear to be solid excreta. They are much smaller than the ova and moreover are spherical." The description and figures, although confused and incomplete, are definite enough to identify the species and validate the specific name.

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Linton (1901) reported *D. pyriforme* from five different species of fishes at Woods Hole: *Palinurichthys perciformis;* the squeteague, *Cynoscion regalis;* the kingfish, *Menticirrhus saxatilis;* the summer flounder, *Paralichthys dentatus;* and the scup, *Stenotomus chrysops.* Specimens from *S. chrysops* were described and illustrated, Figure 346. This figure is consistent with Figure 56 of his (1900) paper.

Linton (1905) recorded *D. pyriforme* from fishes taken at Beaufort, North Carolina, including the menhaden, *Brevoortia tyrannus;* the pinfish, *Lagodon rhomboides;* and the silversides, *Menidia menidia.* There were no descriptions or figures.

Linton (1940) transferred *D. pyriforme* to the genus *Lepocreadium* Stossich, 1904 as *L. pyriforme* (Linton). He stated, p. 84, "To this species are referred certain small distomes which, although differing in many details of structure, resemble each other sufficiently to warrant their inclusion in the same specific grouping when allowance is made for such differences as may be accounted for by varying conditions of contraction and age." The specific diagnosis is so indefinite and general that it might include a group of genera. The specimens, with dates of collection and brief descriptions, were reported from the sand-launce, *Ammodytes americanus;* the harvestfish, *Peprilus paru;* the cutlassfish, *Trichiurus lepturus;* the bluefish, *Pomatomus saltatrix;* and the dollarfish, *Poronotus triacanthus;* in addition to the other species listed earlier, *viz.*, *P. perciformis, C. regalis, M. saxatilis; P. dentatus;* and *S. chrysops. Lepocreadium pyriforme* was depicted by three figures; Figure 47 represents a specimen from *P. perciformis;* the source of Figure 48 is not given; and Figure 49 is of a worm from *P. triacanthus.* The specimens shown in Figures 47 and 49 are probably congeneric but obviously belong to different species. They differ in morphological detail, including relative length of prepharynx and esophagus and of the digestive ceca, and in extent of vitellaria. Linton did not designate a type of *Lepocreadium pyriforme.* Sogandares-Bernal and Hutton (1959) declared, p. 58, "Linton (1940) apparently has a heterogeneous assemblage of species listed under *Lepocreadium pyriforme.* Linton's (1940: Fig. 48) shows prostate cells surrounding the posterior end of the cirrus sac and it well may be that he confused a species of *Opechona* with *Lepocreadium.* Linton (1940) does not mention the presence of an epithelial esophagus. Here again a study of Linton's material is necessary." Sogandares-Bernal and Hutton (1960) discussed the status of some marine species of *Lepocreadium* Stossich, 1904 from the North American Atlantic and examined certain specimens from the Linton collection. The type material was not observed but specimens were depicted from *P. perciformis* (Figure 9), from *A. americanus* (Figure 10), and *Peprilus alepidotus* (Figure 12). They stated, p. 282, "The specimens pictured in figures 10 to 11 differ from the specimen pictured in Figure 9 by possessing a longer post-testicular space and vitellaria extending to cecal bifurcation and shorter prepharynx. The specimen pictured in Figure 12 differs from the specimens pictured in Figures 10 and 11 by possessing a cirrus sac which scarcely extends posterior to the acetabulum as compared with cirrus sac extending posterior to acetabulum by half the cirrus sac length, and from the specimen pictured in Figure 9 by possessing vitellaria which extend to the cecal bifurcation, shorter prepharynx and esophagus almost lacking. *L. pyriforme* should be redescribed if the holotype becomes available. The specimens studied here were not numerous enough to evaluate variation of the species. Figure

9 is identical with Linton's (1940) Figure 47 from the same host. There is little doubt that our Figure 9 and Linton's Figure 47 were drawn from the same specimens collected from the type host, *Palinurichthys perciformis*." Nahhas and Cable (1964) listed a single, juvenile specimen from *P. paru* as *Lepocreadium pyriforme* (Linton, 1900) Linton, 1940, because of its similarity to his (1940) Figure 47. Nahhas and Short (1965) described *Lepocreadium brevoortia* n. sp., from the menhaden, *B. tyrannus,* and distinguished it from all 21 other species in the genus by the massive pharynx and spined cirrus. They stated, p. 43, "*L. pyriforme* (Linton, 1900) Linton, 1940 has a spiny cirrus. Sogandares-Bernal and Hutton (1960) discussed this species and concluded that there are several species involved in Linton's descriptions. Nahhas and Cable (1964) accepted as this species only individuals that are similar to Figure 47 (Linton, 1940) or Figure 9 (Sogandares-Bernal and Hutton, 1960)."

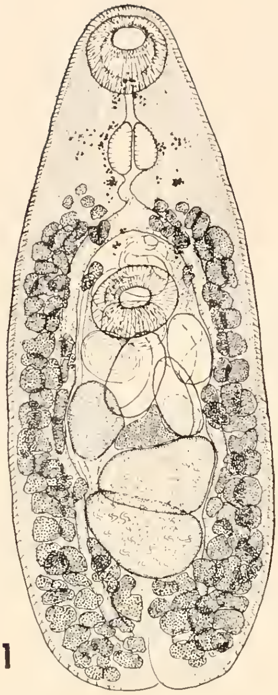
But the status of *Lepocreadium pyriforme* (Linton, 1900) Linton, 1940 remains anomalous. The specimens described and named *Distomum pyriforme* by Linton (1900) are not congeneric with those portrayed in his (1940) Figures 47 and 49 as representative of *Lepocreadium pyriforme*. Many species with divergent morphology have been assigned to *Lepocreadium* and as a result the generic concept has become indefinite and uncertain. Indeed, the genus *Lepocreadium* Stossich, 1904 is not clearly delimited. It was based on *Distomum album* Stossich (1890) from *Cantharus orbicularis* taken at Trieste. This species was included in the genus *Creadium* Looss, 1894 by Looss (1894). But *Creadium* was preoccupied as a generic name and was replaced by *Allocreadium* Looss, 1900. Stossich (1901) described *Allocreadium pegorchis* from *Maena smaris*, taken at Trieste, and he (1904) named the species, *album,* type of a new genus, *Lepocreadium*, in which he included *Lepocreadium pegorchis* (Stossich, 1901), transferred from *Allocreadium*. As diagnostic features of the new genus, Stossich listed: body elongate, cylindrical, rounded posteriorly, attenuated anteriorly; acetabulum at end of the anterior third of body, somewhat smaller than the terminal oral sucker. Digestive tract with prepharynx, robust and elongate pharynx, short esophagus, bifurcation immediately anterior to the acetabulum, ceca extend to posterior end of body. The figure in his (1890) report shows the ceca ending blindly. The testes are subspherical, contiguous, one immediately in front of the other; cirrus sac clavate, with seminal vesicle and spined cirrus. The genital pore is preacetabular, on left side, and the ovary globular, pretesticular, displaced to right of median plane. The vitellaria are well developed, and composed of numerous follicles which extend posteriorly from the level of the acetabulum to the posterior end of the body and become confluent posterior to the testes, and the uterus is short, between ovary and acetabulum; eggs few, large. The species is common in the pyloric ceca and anterior part of the intestine of *Cantharus orbicularis* and *Oblata melanura*.

The life-cycle and developmental stages of the species described as *Distomum pyriforme* Linton, 1900 are described in the present paper. The specimens differ from *Lepocreadium album* in many respects; the bifurcation of the digestive tract is nearer the anterior end; the ceca open into the posterior end of the excretory vesicle; the testes are diagonal, the left testis anterior to the right one; the vitellaria extend from the level of the pharynx to the posterior end of the body, but are not confluent in the median plane. These differences appear of generic significance and, accordingly, *D. pyriforme* Linton, 1900 can not be included in the genus *Lepocreadium.*

The present investigation is an outgrowth of a survey of snails in the Woods Hole area, begun in an attempt to find the cercarial stages of two species of digenetic trematodes whose encysted metacercariae occur in the gills of *Fundulus heteroclitus* and *Fundulus majalis.* Examination of *Anachis avara* yielded an ophthalmotrichocercous cercaria whose body structure recalled the metacercaria from *Pleurobrachis pileus* which the writer had studied at Roscoff, France, thirty-five years before. The morphological agreement was striking. The larvae from *P. pileus,* known as *Opechona bacillaris* (Molin, 1859) have been observed repeatedly in Europe, but the life-cycle has not yet been elucidated. The species was described initially as *Distomum bacillare* by Molin (1859) on specimens from the intestine of *Centrophus pompilius* taken in the Adriatic. It was described by Olsson (1868) as *Distomum increscens* from *Scomber scombrus, Merluccius vulgaris,* and *Hippoglossus maximus* taken on the west coast of Sweden. Stossich (1887) redescribed *D. bacillare* (Molin, 1859) from *S. scombrus* taken at Trieste, but the account was imperfect and omitted certain important features. Odhner (1905) compared specimens of *D. bacillare* from the Adriatic with *D. increscens* Olsson and announced their identity. Lebour (1908) described specimens from the whiting, *Gadus merlangus,* taken off the Northumberland coast of England, as a new species, *Pharyngora retractilis.* The worms were similar to *D. bacillare,* but appeared to be distinct. Nicoll (1910) examined certain of Stossich's specimens of *D. bacillare* and recognized them as identical with *P. retractilis,* which he had found a common parasite of *S. scombrus* and *G. merlangus* in British waters. He redescribed the species as *Pharyngora bacillaris* (Molin, 1859). In the same paper, Nicoll (1910) reported a larval trematode, found in plankton tow, which agreed so completely with juvenile specimens of *P. bacillaris* found in fishes, that he was "practically certain" of their identity. Lebour (1916) reported unencysted metacercariae of *P. bacillaris* in the medusae of *Obelia* sp., *Cosmetira pilosella, Turris pileata* and *Phialidium hemisphericum* and in the ctenophore, *Pleurobrachia pileus,* taken at Plymouth. She (1917) recorded the metacercariae from *Sagitta bipunctata;* also, she described and figured an ophthalmotrichocercous cercaria, taken in tow netting, as the larva of *P. bacillaris.* Ward and Fillingham (1934) described *Opechona alaskenisis* from an unidentified toadfish, taken in Alaska. They recalled that Looss (1907) had designated *D. bacillare* (Molin, 1859) as type of a new genus, *Opechona,* that the publication by Looss was about one year earlier than the paper by Lebour in which she erected the genus *Pharyngora,* and since both generic names are based on *D. bacillare* Molin, 1859, *Opechona* has priority and *Pharyngora* disappears as a synonym.

THE LIFE-CYCLE

It has long been known that certain hydrozoan and scyphozoan medusae of the Woods Hole region harbor unencysted metacercariae of digenetic trematodes. The identity of these larval forms has never been established. In an abstract, Stunkard (1967a) reported natural infections in *Bougainvillia carolinensis, Gonionemus vertens,* and *Chrysaora quinquecirrha.* He also reported an undescribed ophthalmotrichocercous cercaria from *Anachis avara,* whose morphology closely paralleled that of the metacercariae in the jelly-fishes. To test the possibility of specific identity,



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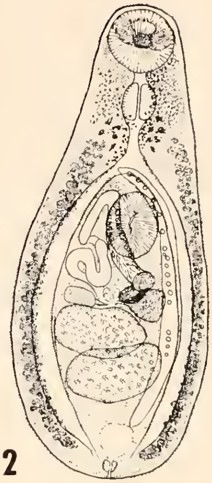

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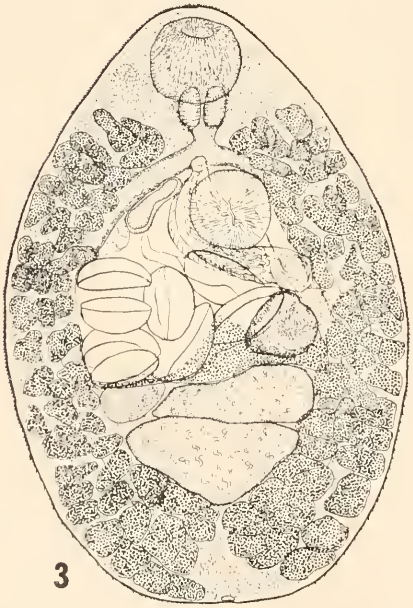

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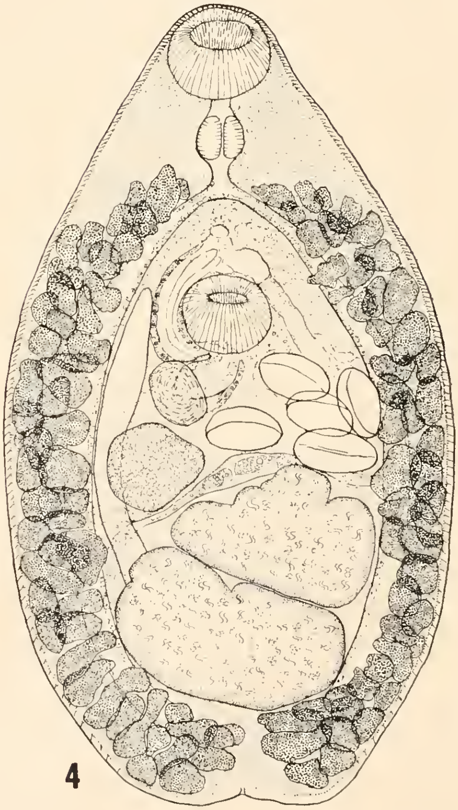

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Figures 1–4.

individuals of *G. vertens* and *C. quinquecirrha* were exposed to these cercariae with resultant massive infections. The specimens of experimental infection were indistinguishable from those of natural infections. The metacercariae in medusae grow very slowly and very little; moreover, they are infective immediately, so the cnidarians are hardly more than paratenic hosts (hôtes d'attente). Early attempts to complete the life-cycle were unsuccessful, since fishes could be maintained for only brief periods in the small aquaria and warm water of the rooms in the Marine Biological Laboratory. Through the kindness of Mr. Charles L. Wheeler, Director of the Woods Hole Aquarium, large tanks with cold water were made available for infection experiments. Experimentally infected medusae, both *G. vertens* and *C. quinquecirrha,* were placed in aquaria with different species of fishes that had been in captivity for long periods. The scup, *S. chrysops,* and the sea-bass, *Centropristis striatus,* were the only species observed to eat the jellyfishes; other species: mackerel, *Scomber scombrus;* sea-robin, *Prionotus carolinus,* and the cunner, *Tautogolabrus adspersus;* apparently ignored the medusae. The fishes were fed four times at five day intervals. Only *S. chrysops* became infected. Four scup were exposed; a sea-bass ate one scup which was in the aquarium with it; the three remaining fishes, dissected four weeks after the first feeding, yielded 92 worms, ranging from juveniles to gravid specimens. One fish contained 47 worms, one 24 worms, and the third 20 worms. The juvenile specimens agree with the type specimens of *D. pyriforme* and the adult worms are so similar to the description and figures (Linton, 1900, Fig. 56; 1901, Fig. 346), that all must belong to a single species. The specimen portrayed in Linton's (1901) Figure 346 was taken from a scup. In the (1967a) report, Stunkard referred the worms to *Opechona* or a closely related genus, but final allocation was deferred pending information on the number and pattern of the flame-cells and penetration-glands of the cercaria.

The study of *D. pyriforme* was continued in the summer of 1968, which provided additional information and data on the excretory system and penetration-glands of the cercaria (Stunkard, 1968). Lebour (1916) reported unencysted metacercariae of *Pharyngora bacillaris* [=*Opechona bacillaris* (Molin, 1859) Looss, 1907] in various medusae and the ctenophore, *Pleurobrachia pileus,* and Stunkard (1932) found these larvae in the same ctenophore at Roscoff, on the Brittany coast of France. Martin (1945) reported unencysted metacercariae in the ctenophore, *Mnemiopsis leidyi,* at Woods Hole. These metacercariae were traced to trichocercous cercariae that developed in sporocysts and emerged from the bivalve mollusk, *Laevicardium mortoni.* Although the metacercariae were not closely related to *Opechona,* the presence of metacercariae in these ctenophores was an item of much interest. Accordingly in 1968, specimens of *M. leidyi* and of the scyphozoan, *Aurelia aurita,* were exposed to the cercariae from *A. avara.* The cercariae swarmed around the ctenophores, penetrated in enormous numbers, and

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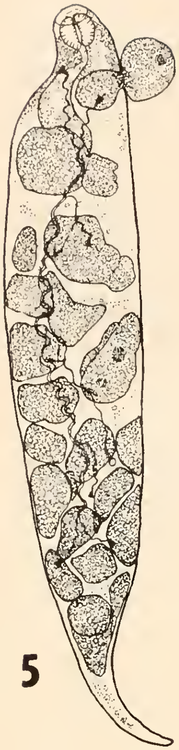
FIGURE 1. Adult worm of *Neopechona,* natural infection, fixed without pressure, ventral view, length, 0.44 mm.

FIGURE 2. Juvenile specimen, experimental infection, fixed well extended, dorsal view, length 0.36 mm.

FIGURE 3. Adult worm, experimental infection, fixed under pressure, dorsal view, length, 0.50 mm.

FIGURE 4. Adult worm, natural infection, fixed under pressure, ventral view, length, 0.66 mm.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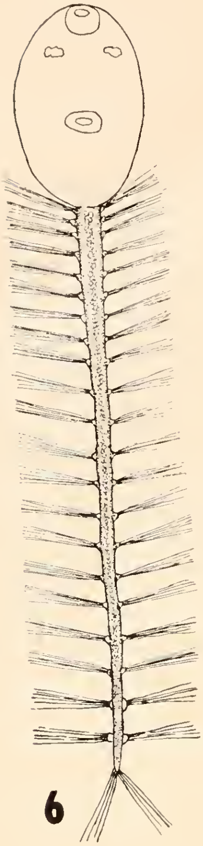
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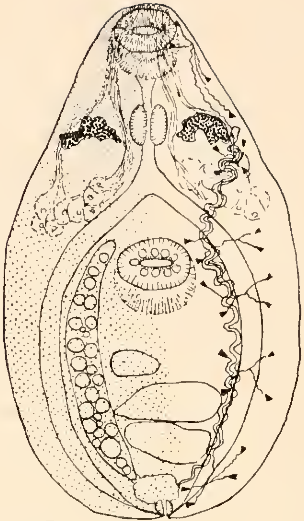
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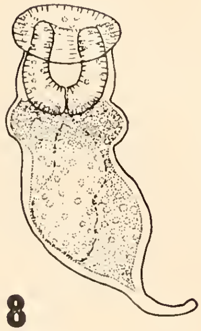
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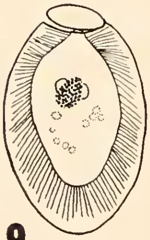
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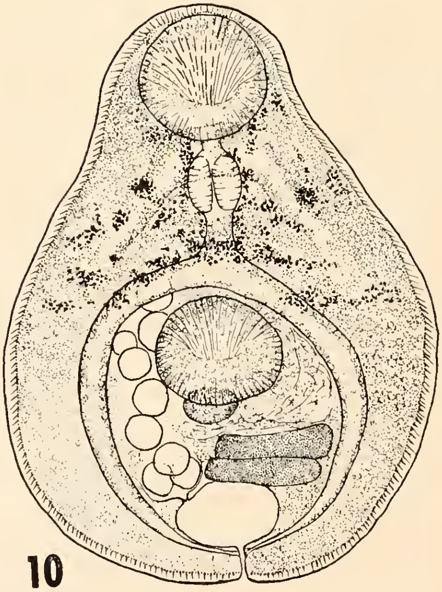
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Figures 5–10.

M. leidyi proved to be a favorable intermediate host. *Aurelia aurita* was not susceptible; the cercariae made no attempt to penetrate this species.

The cercariae are produced in rediae and there are at least two generations of rediae. Most of the cercariae emerge from the snail during the night or early morning, although some are shed during the day. When released from the snail, they swim at all levels of the water, darting about rapidly, with the body retracted and the long tail in advance. On emergence, they tend to accumulate on the dark side of the container, but when older, by late afternoon or next morning, they collect on the light side of the bowl. They attach by the tips of their tails, which precede in locomotion, at any part of the jelly-fish or ctenophore, but more often on the aboral surface. After attachment, the cercaria turns and enters the tissue anterior end first, advancing by movements of both tail and body. The contents of the penetration-glands are extruded during penetration and subsequent migration of the larvae. When the body becomes firmly embedded in the jelly, the lashing of the tail frees it from the body and it continues to swim, sometimes for hours. The cercariae may attach to the bottom of the container by the tips of their tails which contain glandular cells. In the medusae, the metacercariae became uniformly distributed throughout the body (Fig. 11), whereas in the ctenophores they accumulated at the bases of the combs, sometimes as many as three or four between two combs. The factors determining the cause and course of migration are quite unknown.

Eggs from worms of natural infection have been embryonated (Fig. 9); the miracidia develop and emerge in 9 to 10 days at laboratory temperatures. They have ocelli with conspicuous lenses, long cilia, and swim rapidly. A specimen of *Anachis avara* exposed to unincubated eggs on July 17, 1968 was dissected on August 2; it contained two sporocysts. They were oval, about the same size, 0.060 by 0.050 mm, and the largest germ-ball was 0.021 by 0.016 mm. Four snails exposed on July 23 to embryonated eggs from which miracidia were emerging, were dissected on September 1. All were infected, with two to six sporocysts in each. The sporocysts were oval to irregular in form, 0.30 by 0.25 mm to 0.60 by 0.35 mm. The smaller ones contained germ-balls of different sizes; the larger ones contained first generation or mother rediae in addition to germ-balls, and in one snail there were rediae in the haemal sinuses as well as rediae in sporocysts. The rediae in the sinuses were 0.12 by 0.052 mm to 0.145 by 0.042 mm, and the pharynx measured 0.030 to 0.036 mm; so these rediae were approximately the same size as daughter-rediae of natural infections, found free in the haemal sinuses of naturally infected snails.

The data from life-history aid in clarification of the systematic position of the species. When the cercariae from *A. avara* proved to be the larvae of *Distomum pyriforme* the taxonomic problem was simplified. Substantial differences preclude

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FIGURE 5. Redia of *Neopechona,* flattened under coverglass pressure, cercarial germ-ball emerging at birth pore, length, 0.75 mm.

FIGURE 6. Cercaria, outline from sketches of living specimens.

FIGURE 7. Cercaria, morphology from sketches of living specimens.

FIGURE 8. Daughter redia, fixed and stained specimen, 0.12 mm long.

FIGURE 9. Egg, with miracidium, from pencil sketches of emerging larva.

FIGURE 10. Metacercaria, from *Mnemiopsis leidyi,* experimental infection, fixed under coverglass pressure, ventral view, specimen 0.18 mm long.

the allocation of the species *pyriforme* to the genus *Opechona*, and it is designated as type of a new genus, *Neopechona*, in the subfamily Lepocreadiinae.

NEOPECHONA GEN. NOV.

Diagnosis

Lepocreadiidae, Lepocreadiinae. Distomes with ovate to pyriform body, cuticle armed, cirrus and metraterm spinose. Acetabulum preequatorial. Oral sucker readily inverted, about the same size as the acetabulum, prepharynx and epithelial pseudo-esophagus present, ceca long, unite with terminal portion of the excretory vesicle to form the uroproct. Testes two, contiguous, diagonal to almost tandem, in posterior half of body. Genital pore preacetabular, submedian; cirrus sac clavate, extends almost to level of ovary, contains cirrus, prostatic cells and internal seminal vesicle; external seminal vesicle large. Ovary on right side, pretesticular; Laurer's canal and seminal receptacle present; metraterm short, less than the diameter of the acetabulum. Vitelline follicles from level of pharynx to posterior end of body, dorsal, lateral and ventral to ceca, not confluent in the median plane. Vitelline receptacle median, pretesticular; uterus short, winding, pretesticular; eggs few, large, not embryonated. Excretory vesicle tubular, dorsal, on right side of body, extends to level of pharynx, contains spherical concretions. Parasites in digestive tract of marine fishes. Asexual stages in gastropods; metacercariae unencysted in medusae and ctenophores. Type species: *Neopechona pyriforme* (Linton, 1900) new combination. Synonyms: *Distomum pyriforme* Linton, 1900; *Lepocreadium pyriforme* (Linton, 1940) (in part).

Differential diagnosis

Neopechona shares characters with both *Lepocreadium* and *Opechona*. In the key to genera of Lepocreadiinae Odhner, 1905, formulated by Yamaguti (1958), *Neopechona* is closest to *Opechonoides* Yamaguti, 1940. But it differs from that genus in length of ceca, location of testes, position of vitelline receptacle, and extent of vitelline follicles.

DESCRIPTIONS

Adult

A large, living specimen measured without pressure, in contracted condition was 0.60 mm long and 0.052 mm wide; extended it was 1.80 mm long and 0.25 mm wide. Fixed and stained gravid specimens (Figs. 1, 3, 4) measure 0.32 to 0.82 mm in length and 0.25 to 0.40 mm in width. The largest juvenile specimen (Fig, 2) is 0.36 mm long and 0.16 mm wide. The body is pyriform, widest posteriorly, at the level of the testes. The anterior end is mobile; when extended and narrowed, the prepharynx may be as long as the diameter of the oral sucker and the pseudo-esophagus may be almost as long. However, the anterior end is usually not extended and frequently it is retracted with the oral sucker introverted within the anterior end of the body. In normal condition (Figs. 1, 2, 4), both prepharynx and pseudo-esophagus are evident. In young specimens, diffuse pigment, from the disintegration of the ocelli, may be present in the lateral areas at the level of the pharynx. The cuticula is spined throughout, the spines are about 0.005 mm long, imbricate, set closely in the anterior part of the body, becoming sparser posteriorly. The musculature of the body-wall is not strongly developed and in gravid specimens, the reproductive structures are so predominant that mobility is restricted. The acetabulum is situated about one-third of the body length from the anterior end; it is 0.07 to 0.09 mm in diameter, the measurement is influenced by the degree of pressure exerted by the coverglass. The oral sucker is approximately the same size. The pharynx is 0.035 to 0.043 mm in diameter; it is almost spherical although it becomes longer in the anteroposterior axis when the anterior portion of the body is extended. The pseudo-esophagus is lined with epithelium, continuous with that of the ceca. The bifurcation of the digestive tract varies in position with extension and retraction of the body, but is situated a short distance anterior to the acetabulum. The ceca extend posteriorly between the vitellaria on the lateral and the gonads on the medial sides, and open into the terminal part of the excretory vesicle, forming the uroproct.

The excretory system was worked out in cercarial and metacercarial stages and persists without change in the adult condition. In adult worms, the concretions may reach 0.014 mm in diameter.

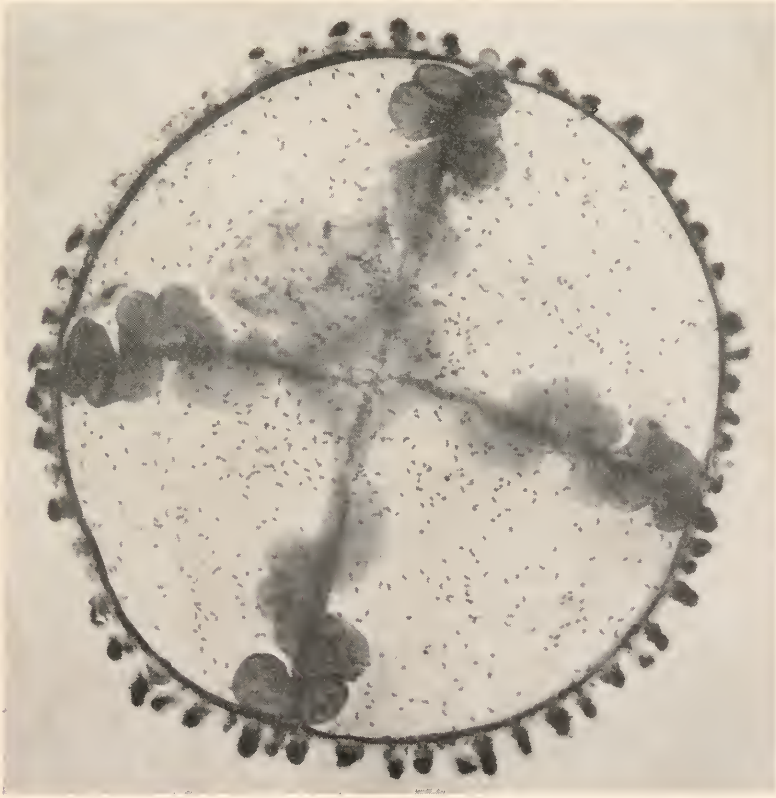

FIGURE 11. Photograph of a specimen of *Gonionemus vertens*, experimental infection of *Neopechona pyriforme*, exposed one day to cercariae; *G. vertens*, 12 mm in diameter.

The testes are situated in the posterior two-fifths of the body, disposed slightly diagonally; typically the posterior testis is slightly larger, and is located on the right side a short distance posterior to the ovary, but their positions are modified by elongation and contraction of the body and by the accumulation of eggs in the uterus. When the body is fully extended, the testes may be almost tandem and when retracted, the organs may be displaced (Fig. 3), in which the anterior testis is shoved toward the right. The testes are notched, but not lobed; they are oval to ovate, longer in the transverse axis, and vary much in size. In mature specimens they measure 0.07 to 0.18 mm in width and 0.04 to 0.09 mm in length. In a large, pressed specimen (Fig. 4), the testes are almost twice as large as in a somewhat smaller specimen (Fig. 1) which was fixed without pressure. In pressed specimens, the worms and their organs appear larger, but the apparent increase in size is the result of flattening. The testes are contiguous and may overlap slightly; sperm ducts arise at the anterior ends and join to form the external seminal vesicle which is continuous with the internal seminal vesicle in the cirrus sac. The external vesicle is usually large, but the size of the vesicles is dependent on the amount of sperm present at any given time. The genital pore is preacetabular, slightly left of the median plane. The cirrus sac is clavate; it extends posteriad in a curved or sinuous course, with the posterior end near the ovary. The internal seminal vesicle is continued by an ejaculatory duct, surrounded by prostatic cells, and the cirrus is eversible. The cirrus and metraterm are spined; the small spines are conspicuous in living specimens, but are not visible in stained and mounted ones.

The ovary is spherical to irregular in shape, 0.04 to 0.07 mm in diameter, sometimes notched, but not lobed, situated on the right side near or slightly posterior to the middle of the body. It is just anterior to the testicular zone and directly ventral to the anterior extension of the excretory vesicle. The oviduct arises at the median, posterior face of the ovary; it passes mediad, receives a duct from the seminal receptacle, gives off Laurer's canal, then receives the common vitelline duct and opens into the ootype, surrounded by the cells of Mehlis' gland. The uterus coils forward between the seminal vesicle and seminal receptacle and posterior part of the cirrus sac, chiefly on the left side of the body; the metraterm is short, about one-half to two-thirds of the diameter of the acetabulum. The vitellaria are extensively developed, extending chiefly in the extracecal fields from the level of the pharynx to the posterior end of the body. Follicles extend dorsal and ventral to the digestive ceca, but are not confluent in the median plane. Longitudinal collecting ducts receive cells from the follicles and, at the postovarian level, ducts from the two sides pass mediad to form the large vitelline receptacle, from which the duct leads to the oviduct. The eggs are thin-shelled, operculate, relatively enormous, 0.056 to 0.062 mm long and 0.036 to 0.040 mm wide, few in number, not embryonated. The operculum is 0.021 mm in diameter. When passed from the worm, the eggs slowly sink in sea water but catch on bits of debris; when embryonated, they are larger, measuring 0.068 to 0.072 mm in length and 0.042 to 0.046 mm in width, contain a bubble of gas and float when loosened. The miracidium (Fig. 9) does not fill the egg-shell; it measures 0.046 by 0.029 mm; the ocellus is 0.007 to 0.008 mm in diameter. The ciliation is uniform except for the apical papilla and the cilia are 0.005 to 0.006 mm in length.

Redia

The haemocoele of a naturally infected snail contains a large number of very small daughter rediae. Figure 8 was made from a specimen that measures 0.13 mm long. In these small rediae, the pharynx is 0.03 to 0.037 mm in diameter, virtually as large as in the large gravid rediae (Fig. 5). The rediae of this species are relatively small, the largest is 0.75 mm long. They are cylindrical, without locomotor appendages, widest near the anterior end, attenuated posteriorly with a curved tail-like tip. The body wall is delicate but in living specimens contraction of the circular muscles may produce one or more constrictions, that may give a redia a collared, neck-like appearance or a dumb-bell shape. The rediae contain orange-yellow pigment and in a few individuals there are pigmented ocelli. The pharynx may be protruded, may be preceded by a ring-like collar, and in many of the fixed and stained specimens, the pharynx is retracted within the anterior portion of the body. The intestine is saccate and very small. The excretory system is double with pores on either side in the posterior portion of the body. From each pore a collecting duct passes anteriad and near the middle of the body it divides into anterior and posterior branches. Each branch has a recurrent tubule and continues toward the corresponding end of the redia where it divides into two tubules. Each tubule terminates in a flame-cell and the excretory pattern (Fig. 5) is identical with that of a very young cercaria. The birth-pore is ventral, near the anterior end (Fig. 5).

Cercaria

The cercariae (Figs. 6, 7) are distomate, ocellate, and trichocercous; they develop in rediae in the haemocoele of the snail, emerge while still immature, and complete their development in the haemal sinuses. The body is oval to pyriform, and may be wider in either the anterior or posterior region. In living specimens, observed under coverglass, the body contracted measured 0.16 mm long and 0.13 mm wide; extended it measured 0.50 mm long and 0.06 mm wide. Under slight coverglass pressure, the bodies of cercariae measure 0.20 to 0.32 mm long and 0.11 to 0.16 mm wide. Fixed in hot whirling fluid, the bodies are 0.15 to 0.19 mm long, 0.085 to 0.095 mm wide and the tails are fully extended, 0.60 to 0.65 mm long. When swimming normally, the body is contracted, almost circular in outline, about 0.12 mm long and the tail is extended, about five times the length of the body. The stem of the tail narrows gradually from base to tip and the median portion is 0.03 to 0.05 mm in width. The tail bears paired setaceous tufts, enclosed in delicate membranes and termed "finlets" by Cable (1954). There are 21 lateral pairs and one terminal pair. The lateral pairs are 0.10 to 0.12 mm long; the terminal ones are 0.073 mm long. The lateral pairs are flattened anteroposteriorly and appear as oars or paddles, which make the tail a powerful swimming organ. Each lateral finlet has a row of five or six rays, rod-like or tubular supports, in dorsoventral alignment. They appear to be formed by a secretion; when the tail disintegrates, the finlets separate from the tail-stem and a widened portion of each ray migrates from the base to the tip where it appears as a minute refractive spherule. Each terminal finlet has only three rays. Under high magnification, the bases of the rays appear multiple, as though three, four, or more strands had fused to form each rod-like support.

On the body, but not the tail of the cercaria, the cuticula contains retrorse spines, arranged in an imbricate pattern. The acetabulum is situated at or near the middle of the body; it is 0.045 to 0.054 mm in diameter, and the opening bears nine papillae (Fig. 7). The ocelli consist of aggregates of irregularly disposed pigment, typically longer in the lateral axis; they are at the level of the pharynx or slightly anterior to it and about the same size as the pharynx.

The oral sucker is spherical to pyriform, often slightly wider in the posterior half; it measures 0.055 to 0.060 mm in diameter. The pharynx is 0.016 to 0.024 mm in diameter; the prepharynx and pseudo-esophagus vary in length as the anterior end of the body is extended and retracted, and either may be as long as the pharynx. The digestive tract bifurcates a short distance anterior to the acetabulum. The pseudo-esophagus is lined with epithelium, continuous with that of the ceca. The ceca extend laterally and caudally, opening into the posterior end of the excretory vesicle.

There are eight pairs of penetration-glands, situated in the lateral areas between the ocelli and the level of the acetabulum. On either side, ducts from three cells pass forward, lateral to the ocellus and dorsal to the excretory tubules, while ducts from the other five cells pass mediad of the ocellus and around and above the oral sucker. All ducts open to the surface at the anterior end of the cercaria, above the opening of the oral sucker. The glands are spherical to oval, about 0.010 mm in diameter, often partially superimposed. They become oval to elongate under pressure as the secretion passes from the body of the cell into the duct. The primordia of the gonads are clearly recognizable, situated in the caudal one-fourth of the body.

The excretory system does not extend into the tail. In the young cercaria it is double, with pores on either side, near the posterior end of the body, and collecting ducts that extend forward and contain tufts of cilia as they turn posteriad. After a short recurrent portion, each collecting duct divides into anterior and posterior branches. Each branch gives off a recurrent tubule and later divides to form two terminal tubules; each tubule terminates in a flame-cell and the formula is $2\,[(1+1+1)+(1+1+1)]$. Later, a constriction at the level of the excretory pores cuts off the tail and the posterior portions of the collecting ducts fuse in the median plane to form the primordium of the excretory vesicle. At the same time, the posterior ends of the digestive ceca unite with the caudal portion of the excretory vesicle to form the uroproct. From the anterior end of the fused portions of the collecting ducts, a median dorsal extension develops, forming a thin-walled reserve excretory vesicle, which becomes filled with fluid and may extend to the level of the pharynx. This vesicle later contains concretions, usually disposed in a linear series, but sometimes, on retraction of the body, they may be in a zigzag pattern or in a double row. The concretions vary in size from granules or globules to concentric layers of refractive material as much as 0.14 mm in diameter. The portion of the median vesicle immediately anterior to the uroproct forms a pulsatile bladder, separated by sphincters from the uroproct and the more anterior portion of the vesicle. On either side of the pulsatile bladder, a collecting duct, lined with cilia, passes forward, lateral to the digestive cecum. Anterior to the acetabulum the collecting duct divides into anterior and posterior branches. The anterior branch extends almost to the ocellus of that side where it divides to form a duct that passes forward and a duct that turns backward. The anterior duct passes forward, gives off a recurrent tubule that leads to a flame-cell situated just anterior and lateral to the ocellus, then a tubule to a flame-cell located just posterior to the oral sucker, and divides to supply two flame-cells that are lateral to the oral sucker. The backward branch gives off a duct that supplies a group of four cells and continues posteriad where it gives rise to a third group of four cells. In each group, the tubules and flame-cells are disposed in the same manner as described for the most anterior one. In the posterior half of the body, the backward branch of the collecting duct divides forming a pattern that is just the obverse of that in the anterior half of the body. The flame-cell formula of the mature cercaria is $2\ [(4+4+4)+(4+4+4)]$, with six groups of four cells on each side of the body. The most anterior group supplies the preocellar area; the second group the postocellar area; the third group the acetabular area; the fourth group the postacetabular, pretesticular area; the fifth group the testicular area; and the last group supplies the posttesticular region.

Metacercaria

The metacercariae (Figs. 10, 11) are unencysted and mobile; they are only slightly larger than the cercariae. The pigment of the ocelli becomes dispersed, but otherwise there is little change from the cercarial condition.

DISCUSSION

The discovery of the life-history of *N. pyriforme* and its systematic relations adds another link in the chain of evidence that integrates life-cycles, larval stages, developmental features and adult morphology with taxonomy of the digenetic trematodes. The group of trichocercous cercariae was proposed by Lühe (1909) for those species which have the "Schwanz mit Borsten besetz (marin)." These larvae have long been known; *Cercaria setifera* was described by O. F. Müller (1786) but there is no assurance that it is identical with *Cercaria setifera* Joh. Müller (1850). This species, described by Müller, was named by Moulinie (1856). However, it is certain that *Cercaria setifera* of Monticelli (1888, 1914) is not conspecific with that of Müller, 1850. The first suggestion concerning the life-history of the trichocercous cercariae was made by Odhner (1914) who, on the basis of morphological agreement, asserted that *C. setifera* of Monticelli is the larva of *Lepocreadium album* (Stossich, 1890) Stossich, 1904. Dollfus (1925) noted that *C. setifera* of Monticelli is distinct from *C. setifera* Müller, 1850; he gave a summary of the trichocercous larvae known at that time and divided the marine species into two groups, one in which ocelli are present and one in which they are absent. Commenting on this arrangement, Cable (1954) observed, p. 18, "subsequent studies have revealed that such a distinction may be an artificial one, for instances are known in which one cercaria may be ocellate whereas another larva in the same family lacks eye-spots. On the basis of known life histories, it is certain that the trichocercous cercariae listed by Dollfus have adults belonging to three distinct families, the Lepocreadiidae, the Monorchiidae, and the Fellodistomatidae. Furthermore, the last two groups and perhaps all three have some larvae that are not trichocercous. Thus in distinguishing the larvae of these families, the morphology of the body and type of molluscan host are more dependable than is the structure of the tail which can be positively misleading.

Of the non-ocellate cercariae listed by Dollfus, *C. setifera* Müller *nec* Monticelli (the larva of *Bacciger bacciger* according to Palombi (1934a), *C. villoti, C. pelseneeri, C. chiltoni,* and *C. pectinata* Huet *nec* Chilton may be assigned to the Fellodistomatidae." Dollfus recognized disparity among trichocercous cercariae; he (1927) reported an unnamed setigerous larva taken in plankton near Cherbourg, France, stated that it is not identical with *C. setifera* of the Mediterranean, and expressed the opinion that not all trichocercous cercariae are members of *Lepocreadium.* Indeed, Lebour (1917) had described and figured a trichocercous cercaria found in plankton at Plymouth, England, as identical with the unencysted metacercariae in medusae and ctenophores which she (1916) had recognized as larvae of *Pharyngora bacillaris* (Molin, 1859), a common parasite of *Scomber scombrus* and *Gadus merlangus.* Palombi (1934a) announced that *C. setifera* Müller, 1850 develops in sporocysts in *Tapes decussatus;* that the metacercariae occur in the amphipod, *Erichtonius difformis,* and that the adult is *Bacciger bacciger* (Rudolphi, 1819) Nicoll, 1914, a parasite in various members of the genus *Atherina.* As synonyms of *B. bacciger,* Yamaguti (1958) listed *Cerceria lata* Lespès, 1857; *C. lutea* Giard, 1897; and *C. pectinata* Huet, 1891. Palombi (1934b, 1937) reported that *C. setifera* Monticelli, 1914 (in part) *nec* Müller, 1850, is the larva of *Lepocreadium album* (Stossich, 1890). The cercariae are produced in rediae in *Nassa mutabilis,* encyst in *Aplysia punctata* and *Tapes* spp., and become adult in various species of fish. This species was studied by Arvy (1953) who declared, p. 298, "Vue à des stades divers, sur des hôtes variés, dont je me suis appliquée à relever la liste, *Cercaria setifera* Monticelli a reçu des noms divers, mais il semble bien que, dans tous les cas auxquels j'ai fait allusion, il s'agisse de la même larve d'Allocreadiidae." Martin (1938) showed that *Cercaria setiferoides* Miller and Northup, 1926, produced in rediae in *Nassarius obsoletus* of the Woods Hole, Massachusetts area, is the larval stage of a species of *Lepocreadium.* He reported penetration and encystment in the turbellarian, *Procerodes warreni,* and in spionid annelids, although he predicated that these experimental hosts may not be the natural ones. The discovery of the life-cycle and larval stages of *Lepocreadium pegorchis* (Stossich, 1890) by Bartoli (1967) demonstrates the variation in cercarial morphology that may occur in species of a single genus. The cercariae are produced in rediae in *Nassa mutabilis,* but differ distinctly from those of *L. album* (Stossich, 1890), found in the same host, and from *L. setiferoides* (Miller and Northup, 1926) which develop in *Nassarius obsoletus* in America. In *L. pegorchis,* the tail of the cercaria is much shorter than the body, is only weakly functional, and the larvae seldom leave the substratum. They are drawn in the inhalant siphons of various lamellibranch mollusks, where they penetrate the tissues but do not encyst.

A comprehensive account of marine cercariae was given by Holliman (1961) who attempted to assign them to systematic categories. Dollfus (1963) listed the Palearctic and Indian marine coelenterates which harbor larvae of digenetic trematodes. The hosts are mainly planktonic and manifest little specificity. He noted that cercariae of the Lepocreadioidea are produced in rediae, have ocelli, and do not encyst. Accordingly, since Palombi (1937) reported encystment of metacercariae of *L. album,* Dollfus suggested that he may have been dealing with more than one species. Consideration of the life-cycles of digenetic trematodes with correlation of intermediate and final hosts, of larval stages, cercarial types, and systematic relations, discloses one of the enigmas of theoretical zoology. Commenting on the situation, Stunkard (1967b) predicated, p. 675, "The course of development reflects, to a considerable extent, the evolutionary history of the species. In endoparasitic animals, the individual perishes with the host and larval stages provide for dispersal and perpetuation of the species. The origin and significance of larval stages find their explanation in the life-cycles of the species in which they occur. According to Leuckart (1891a) larval structures are adaptations for earlier independent existence of offspring with consequent increase in reproductive capacity. Larval adaptations may be as complete and perfect as those of adult individuals." Stunkard reasoned that the digenetic trematodes were derived from turbellarian-like ancestors, that they became parasites of mollusks in Cambrian time, and that with the advent of fishes and their ingestion of mollusks, the sexual stages of the worms were deferred to the definitive hosts while former hosts were relegated to an intermediate status. Mollusks still serve as first hosts and harbor the initial stages in the life-cycle.

Summary

The digenetic trematode described by Linton (1900) as *Distoma pyriforme* has been reported from many hosts and several species have been included in the accounts, with resultant confusion. It was included in the genus *Lepocreadium* Stossich, 1904 by Linton (1940), but it is not congeneric with *L. album* (Stossich, 1890), type of the genus. Its life-cycle has been elucidated; *Anachis avara* is the first intermediate host, where cercariae are produced in rediae. The cercariae are ophthalmotrichocercous, swim actively with the tail in advance. They penetrate but do not encyst in certain hydrozoan and scyphozoan medusae and in the ctenophore, *Mnemiopsis leidyi.* Developmental and adult stages resulted from ingestion of metaceracariae by the scup, *Stenotomus chrysops.* Eggs from worms were embryonated; miracidia emerged in 8 to 10 days, penetrated into *A. avara,* transformed into sporocysts, and produced rediae in 5–6 weeks. Worms recovered from *S. chrysops* are assigned to a new genus, *Neopechona,* and redescribed as *N. pyriforme* (Linton, 1900) new combination. The genus is included in the subfamily Lepocreadiinae Odhner, 1905.

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OSMOREGULATORY CAPACITIES OF *CALLIANASSA* AND *UPOGEBIA* (CRUSTACEA: THALASSINIDEA)[1]

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The thalassinid burrowing mudshrimps are an example of a group of decapod Crustacea about which relatively little is known with respect to osmoregulatory capacities. A few pertinent physiological studies have yet to become common coin of the scientific literature. Thus, like several invertebrates of the Black Sea, *Upogebia* (=*Gebia*) *littoralis* was shown to regulate its osmotic concentration in brackish waters (Zenkewitch, 1938). Recent unpublished research demonstrated the strong osmoregulatory capacities of the African mudshrimps *Upogebia africana* and *U. capensis* (Hill, 1967). On the other hand, it has been reported that *U. affinis* of eastern North America survived dilutions of seawater (SW) only to a very limited extent (Pearse 1945). Limited tolerance to diluted SW was also indicated for *Upogebia* sp. and *Callianassa affinis* of western North America (Gross, 1957). Although no data were given for thalassinids in his study, Gross concluded that both species are osmo-conformers. Subsequently the genera *Callianassa* and *Upogebia* have been characterized as "polystenohaline" Crustacea with ionic and volume regulation but with little or no osmoregulation (Brown and Stein, 1960; Lockwood, 1962; Kinne, 1963).

As burrowers in marine sediments since at least the Cretaceous Period (Milne Edwards, 1861; Borradaile, 1903), callianassid and upogebiid crustaceans are widely distributed and such distributions include estuarine or other brackish situations (Schmidt, 1921; de Man, 1927, 1928; Pohl, 1946; Day, 1967). The distribution of *Callianassa filholi* of Australia and New Zealand suggests that this species may well experience brackish conditions (Devine, 1966). *C. turnerana* of Africa migrates annually or semi-annually from brackish water bays up fresh-water (FW) rivers and streams, a phenomenon so marked that the Cameroons derives its name from it (Monod, 1927). *C. kraussi* of Africa has a known salinity range of 1.25–59.5‰ (Day, 1951). Both *C. californiensis* and *U. pugettensis* survived the storm inundation of Newport Bay, California (MacGinitie, 1939). Such accounts suggest the euryhalinity of those thalassinids concerned but published physiological evidence for the osmotic capacities of thalassinid Crustacea is generally lacking.

This study compares osmoregulatory capacities in *Callianassa californiensis, Upogebia pugettensis* and *U. affinis*. Preliminary results for *C. filholi* of New

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Zealand are included. In conjunction with a study of metabolic adaptation (R. K. Thompson and Pritchard, *Biol. Bull.*, in press), this report contributes physiological information on a systematically unique group of Decapoda having both anomurous and macurous affinities (Borradaile, 1903; Gurney, 1938; Waterman and Chace, 1960).

MATERIALS AND METHODS

Studies on *C. californiensis* and *U. pugettensis* took place at the Marine Science Center of Oregon State University at Newport, Oregon, during summer, 1966. Animals were collected from the exposed flats of the Yaquina Bay estuary at low tides, transported directly to the laboratory and introduced into holding tanks provided with continuously flowing unfiltered SW. We used animals indiscriminately as collected, excepting those damaged, moribund or in postmolt. Postmolt individuals are recognizable by the softer than usual integument, lighter coloration and cleaner appearance.

Individuals were isolated in compartmentalized aquaria and stepwise acclimated by 20% or 25% SW decrements for 1-day periods, followed by a 3-day acclimation period at the final test salinity. Animals were also introduced directly into 125% SW from the holding tanks and held 3 days. The suitability of the 3-day acclimation interval was established by a study of the time required to reach a new steady state with respect to Cl^- (*cf.* Results). Temperatures of media varied from 13–16° C. Salinity of the laboratory SW for the months June-August, 1966, varied between 33 and 35‰; a salinity of 35‰ was taken to represent 100% SW. Concentrated SW was prepared by means of an electric fan and circulating heater. In this fashion several liters of 175% SW could be prepared within 24 hr and boiling was avoided. In all cases dilutions were made with de-ionized glass-distilled water.

Body fluids were obtained as follows. Animals were removed from the medium, rinsed briefly with distilled water and blotted "dry." Blood was taken by penetration of the thin membrane just posterior to the 5th periopods, using a drawn-out Pasteur pipette. Subsampling and analyses usually proceeded at once, as below. Flame photometer analyses were done at the Department of Zoology, Corvallis. In these cases, diluted blood samples were held overnight at 2° C in 2-ml beakers sealed with Parafilm to restrict evaporation. Urine issuing from the nephropore of the antennal base was collected on Parafilm triangles. The most successful stimulus to micturition was to touch the urinary papilla with a warm blunt probe. This procedure eliminated the possibility of puncture, assuring that the fluid sampled was urine. After at least 0.05 ml was collected, samples were taken from the drop for Cl^- and total osmotic concentration using capillary pipettes (Drummond "microcaps") and Hamilton microliter syringes, respectively. Blood, media and standards were handled similarly. Blood was not filtered.

Body fluid Cl^- was determined electrometrically by a Buchler-Cotlove chloridometer. Cations were determined by a Coleman flame photometer, model 21, with filters for Na^+, K^+, and Ca^{++}. Total osmotic concentrations were determined by the Hewlett-Packard vapor pressure osmometer, models 301A and 302. Salinity was measured either by chloridometer or chemically (Schales and Schales, 1941). Salinities were determined on laboratory SW and habitat water samples. The apparatus used to obtain burrow and interstitial water samples is described elsewhere (R. K. Thompson and Pritchard, in press).

Upogebia affinis was collected near Cape Lookout in the vicinity of Beaufort, North Carolina, on 5 September 1967 (water temperature 22° C) and airmailed to the University of California Bodega Marine Laboratory where acclimation at 22° C commenced at once. Twenty-seven of 36 animals survived the mailing. Animals were transferred to small plastic trays containing 500 ml of medium, 4 per tray. Water was changed daily and aeration was not provided. Animals were stepwise acclimated by 15%–25% SW decrements for 24 hr and allowed to remain at the final test salinity 48 hr. Sampling and analytic methods, where applicable, are as above. All *U. affinis* specimens were sampled for blood twice in the course of experiments. Data from the first sampling, reported here, represent individuals which had not molted up to the time of sampling. After sampling, individuals were replaced in their appropriate medium in order to observe longer-term effects of reduced salinity. Within 4 weeks all individuals molted once and 3 twice. Data from a second sampling, not reported here, represent postmolt individuals. The thalassinids of this study may be serially sampled in the manner described without apparent ill effect as long as the blood volumes withdrawn are not excessive. However, all data reported here derive from individuals without prior sampling history.

Callianassa filholi was collected at Little Papanui Beach of the Otago Peninsula, New Zealand, on 19 May 1968 (water temperature 11.5° C) and air freighted to the Bodega Marine Laboratory. Eleven of 12 animals survived the mailing and were immediately isolated and introduced into flowing 100% SW. Except for lowered temperature (11–13° C), acclimation methods, sampling and analytic procedures are as for *U. affinis*.

RESULTS

Ecology

C. californiensis is abundant throughout the tidal mudflats of Yaquina Bay, particularly on the south banks, confined to a muddy sand zone corresponding roughly to the 0 to +1 foot tide level. That they are more abundant on the south shore is apparently because of the predominance of sandy beds there. Where such beds extend as spits to lower tidal levels, *Callianassa* commonly occurs, indicating no necessary restriction to the higher intertidal. Near the junction of the Yaquina River with the bay, the abundance of *Callianassa* falls off strikingly, and it is scarce or absent from the river system. A single *C. californiensis* was recovered in McCaffery Slough but the species was not found at several collecting localities within a further distance of 1.8–1.9 kilometers above McCaffery Slough. Thus in the overall estuarine system, the *Callianassa* populations (including those of the congeneric *C. gigas*) appear restricted to the bay.

The beds of *C. californiensis* in the Yaquina Bay system contain numerous apparent openings to the surface. Inspection of the openings, however, revealed that they are without exception occluded with substrate during the period of tidal exposure. Careful digging and probing confirmed the absence of well-formed burrows. In general *C. californiensis* is not found within the top 45 cm of substrate at these times but is abundant at or near the prevailing sub-surface low water line. These findings are somewhat anomalous, for as one walks over such a bed, watery upwellings spout from the "openings," suggesting burrows. MacGinitie (1934) has described the burrowing activity of *C. californiensis* and has figured a burrow by means of an observation chamber.

When present, the burrows of *C. californiensis* may be regarded as artifacts of recent burrowing activity. At low tides burrows are not buoyed by SW and, in the absence of wall reinforcement, burrows readily collapse. As recently turned-over substrate, however, occluded burrows offer relatively less resistance to the passage of water. This accounts for the spouting seen when pressure is applied by walking over a bed, and also for the relatively rapid drainage of *C. californiensis* beds at low tide (Stevens, 1928). The present findings indicate that (1) patent openings to the surface generally are absent, and (2) the burrow system is relatively impermanent in the natural environment at Yaquina Bay.

U. pugettensis is abundant throughout the Yaquina estuary wherever suitable substrate is present. Within the bay proper the distribution along the northern shoreline is correlated with the presence of alluvium ranging from muddy silt deposits at the Newport Marina to mud-clay of the sloughs. *Upogebia* is generally absent from the sandier southern shoreline of the bay. Unlike *Callianassa, Upogebia* is widespread within the Yaquina River system up to at least Johnson Slough (*ca.* 7 kilometers beyond Yaquina Bay). Within the bay *Upogebia* is most frequently collected at lower tidal levels at the approximate 0 to −1 foot zone; within the river system *Upogebia* is collected from the shallow muddy gravel near-shore areas, the muddy shoreline and high banks of muddy clay along the

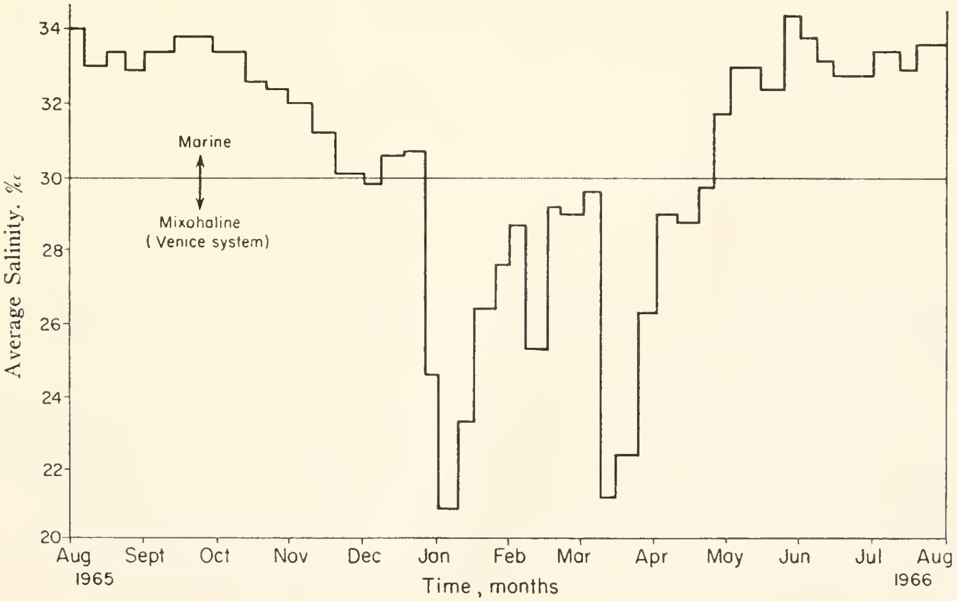

FIGURE 1. Average salinity of Yaquina Bay, Newport, Oregon, for the period August, 1965, to August, 1966. Data through June, 1966, courtesy of the Oregon Fish Commission. See text for explanation.

shoreline, indicating no necessary restriction to the lower tidal zone.

The burrow of *U. pugettensis* has been described (Stevens, 1928; MacGinitie, 1930; R. K. Thompson and Pritchard, in press). We remark only that galleries of the burrow system may penetrate much deeper in the Yaquina region. It is concluded that *U. pugettensis* inhabits a relatively permanent burrow system generally open at the surface.

Salinity of the Yaquina Estuary

Salinity determinations are performed daily at the Marine Science Center. Average salinities for the year 1965–6 are shown in Figure 1. In all cases the data represent bottom salinities of the Yaquina Bay salt water wedge from which the laboratory derives its SW. The intake of the SW system is located approximately where the estuary debouches into the channel leading to the ocean. Salinity values are represented by bars, themselves 7–10 day means of determinations done from once to 19 times within a 24-hr period. In general the smaller number of determinations correspond to periods of stable high salinity and were performed at or near tidal extremes. At times of low and fluctuating salinity, analyses were performed several times daily.

TABLE I

Salinities of water samples taken 11 March 1967 at Yaquina Bay, Newport, Oregon. Surface pool and burrow water samples taken at Coquille Pt. Interstitial water samples taken on the south shore of Yaquina Bay near the laboratory. Values are averages of duplicate determinations

Sample	Depth	Salinity, ‰
Marine Science Center Laboratory SW		28.9
Surface pool # 1		22.5
# 2		22.0
Bay water, Coquille Pt.		22.7
Upogebia burrow # 1	19 in	22.4
# 2	24 in	22.0
# 3	24 in	22.5
Callianassa interstitial		
water # 1	24 in	27.8
# 2	21 in	29.0

It is seen that the periods August-October, 1965, and June-August, 1966, had stable high salinities of 33–34‰. During the summer of 1966 there was negligible rainfall in the Yaquina region, and salinities reflected full strength SW during this time. The periods October, 1965 to January, 1966, and May, 1966 may be regarded as times of gradual decrease and increase of salinity, respectively. The period January-April, 1966 was a time of prevailing brackish conditions (Venice System) (Smith, 1959) since the mean weekly salinity varied in the range 21–29.5‰. It may be expected that bottom salinities relatively close to the ocean would not correspond to those of the mudflats further up the estuary during a brackish and labile period. Other studies of the Yaquina estuary show differences between top and bottom salinities (Burt and McAlister, 1959) and a progressive drop in salinity further up the bay and river system (Frolander, 1964). Quite probably, therefore, animals further up the estuary experience lower salinities than those shown in Figure 1.

Burrow, interstitial and shore water samples (the latter taken at water's edge during ebb tide) were taken periodically from Newport collecting localities during summer, 1966, and salinities determined. Results indicate that without exception resident populations of thalassinids experienced 100% SW during the summer months. On March 11, 1967, following a period of rainfall, samples were again taken at the same Newport localities. Salinity determinations show (Table I) that both species experience brackish conditions. In addition the salinities of samples taken from the burrows of *Upogebia* correspond to those of nearby shore water and surface pools, and that in this particular instance, *Upogebia* experienced relatively more reduced salinity than did *Callianassa.*

Experimental acclimation times

Acclimation times were estimated from experiments measuring the time required to achieve a new steady state with respect to Cl^-. Animals previously acclimated to 100% SW were acutely introduced into 50% SW (Cl^- adjusted to 285 meq/l). Blood Cl^- was monitored for a period in excess of 2 days (Fig. 2). Mean values for the blood Cl^- of the *C. californiensis* and *U. pugettensis* controls kept in 100% SW ($Cl^- = 562$ meq/l) and sampled at the same time as experimentals are between

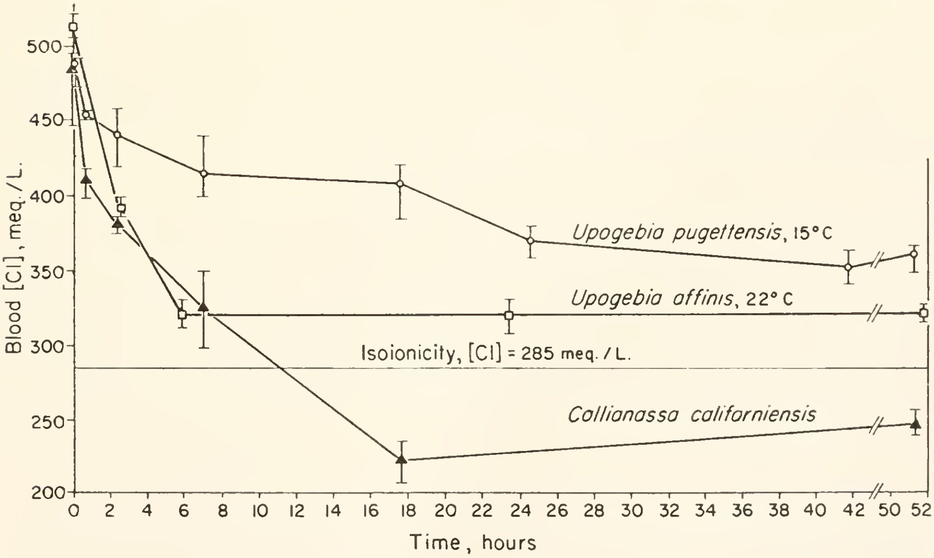

FIGURE 2. Blood chloride concentrations of *Callianassa* and *Upogebia* as a function of time, following acute introduction of animals adapted to 100% SW into 50% SW. ▲ = mean of *Callianassa* Cl, n = 5 at each point; temperatures varied between 13° and 16° C. ○ = mean of *U. pugettensis* Cl, n = 7 at each point. □ = mean of *U. affinis* Cl, n = 3 at each point. Ranges are indicated by vertical bars.

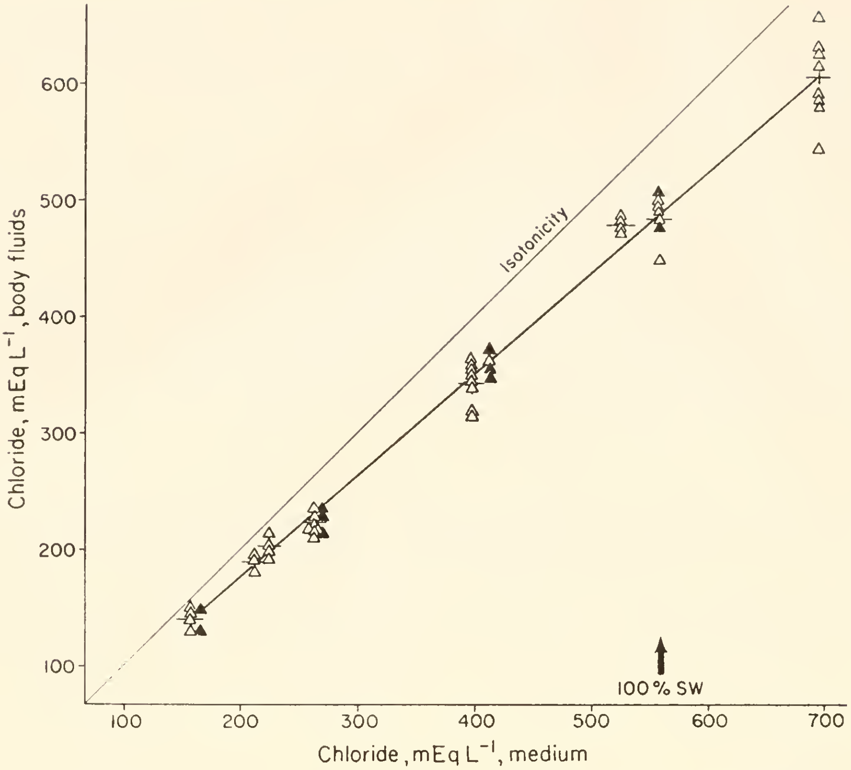

FIGURE 3. The chloride of *C. californiensis* blood and urine as a function of medium chloride concentration. △ = blood; ▲ = urine. Points are averages of duplicate determinations. Horizontal lines indicate means. Temperature of media = 14°–16° C.

480–485 meq/l; the comparable mean for the blood of *U. affinis* is 516 meq Cl$^-$/l. It is seen that the blood Cl$^-$ of *Callianassa* falls most rapidly but stabilizes after 18 hr at a level appreciably hypo-ionic to the medium. Both species of *Upogebia* strongly regulate with respect to Cl.$^-$ Levels of regulation are higher for *U. pugettensis* and the time to reach a steady state longer (42–52 hr). Note that the unequal acclimation temperatures reflect those of the time of capture. We set acclimation intervals for *C. californiensis, U. pugettensis* and *U. affinis* at 3, 3 and 2 days, respectively.

Regulation of chloride

Blood and urinary Cl$^-$ relationships were determined for animals acclimated to media ranging from 10%–125% SW. The blood Cl$^-$ of *C. californiensis* is consistently hypo-ionic over the range 30%–125% SW (Fig. 3). Urine is iso-ionic to blood with respect to Cl$^-$. We were unable to collect urine from animals acclimated to 125% SW.

Both species of *Upogebia* regulate Cl$^-$ (Fig. 4). Both are iso-ionic, or nearly so, in 70% SW ($Cl^- = 395$ meq/l). Between 70%–125% SW Cl^- concentrations for both species are demonstrably hypo-ionic with respect to the medium, and the extent of this hypo-regulation is greater in *U. pugettensis.* Below 70% SW *U. pugettensis* and *U. affinis* strongly regulate the blood concentration of Cl^-, and there appears to be little, if any, difference between the species with respect to the level of regulation. The urine of *U. pugettensis* is iso-ionic to blood with respect to Cl^-.

Mortality and lower lethal limits

The several species proved hardy in the laboratory. In several experiments 3 of 120 *C. californiensis* died in the course of acclimation to media in the range 30%–125% SW, while none of the 11 *C. filholi* died in the stepwise acclimation down to 40% SW. Seven of 147 *U. pugettensis* died in the range 15%–125% SW. Groups of *C. californiensis* and *U. pugettensis* were maintained without loss in 50% SW for more than 3 weeks. Among 27 *U. affinis* there was no mortality in

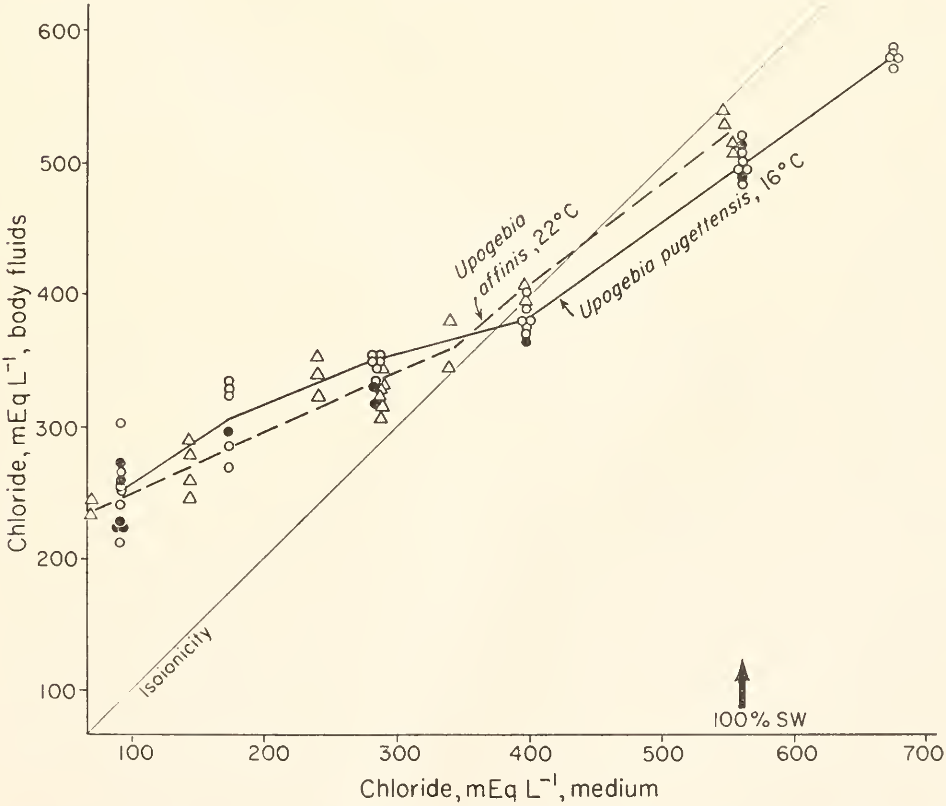

FIGURE 4. The chloride of *Upogebia* blood and urine as a function of medium chloride concentration. *U. pugettensis* blood (○), urine (●); *U. affinis* blood (△). Points are the averages of duplicate determinations.

media ranging from 10% SW to 100% SW. Mortality became pronounced only at salinities less than the lower limits.

Between 25% and 30% SW represents the lower lethal limit for *C. californiensis* under laboratory conditions. Seven animals previously acclimated to 40% SW perished overnight in 25% SW without aeration; 20 animals previously acclimated to 30% SW died overnight in 25% SW with aeration. On the other hand, *C. californiensis* in several experiments survives 30% SW for at least 3 days when provided with aeration, although sluggishness and weakness are manifest. In 125% SW 8 of 9 animals survived 3 days but we did not pursue the upper lethal limit.

U. pugettensis survived 3 days in 15% SW (n = 10) but appeared sluggish;

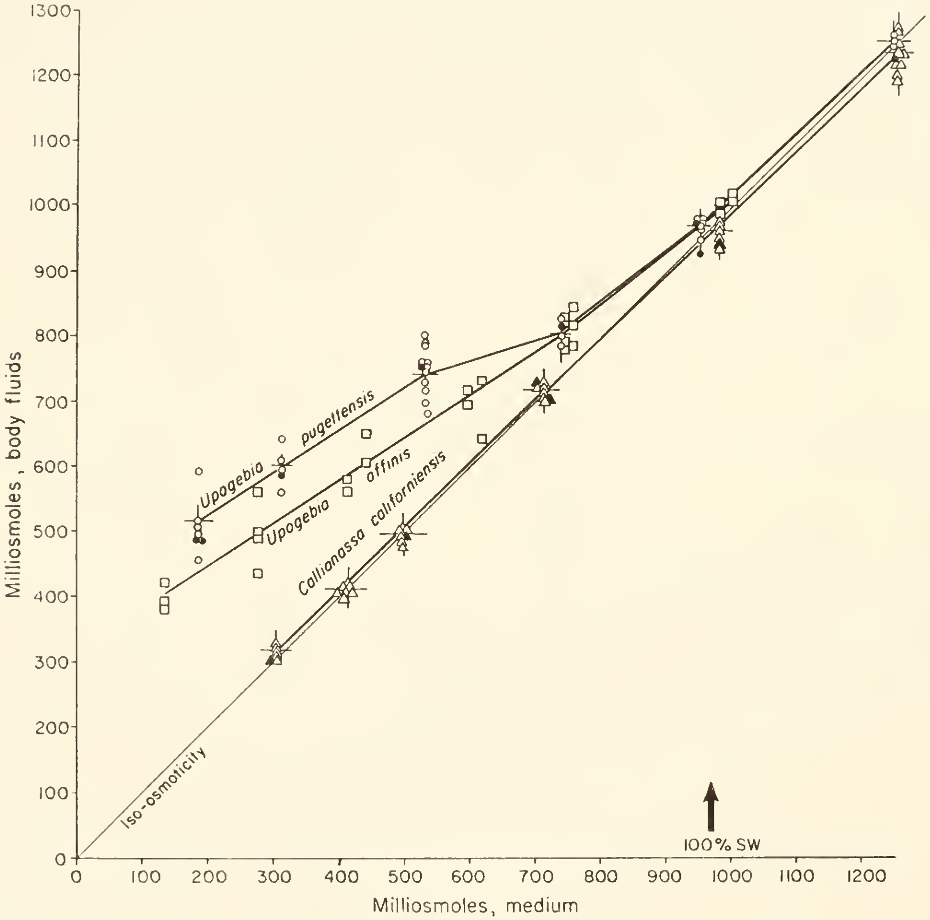

FIGURE 5. Total osmotic concentrations of *Callianassa* and *Upogebia* blood and urine as a function of medium osmotic concentration. *U. pugettensis* (temp. 15° C) blood = ○, urine = ●. *U. affinis* (temp. 22° C) blood = □. *C. californiensis* (temp. 14°–16° C) blood = △, urine = ▲. Means indicated by +.

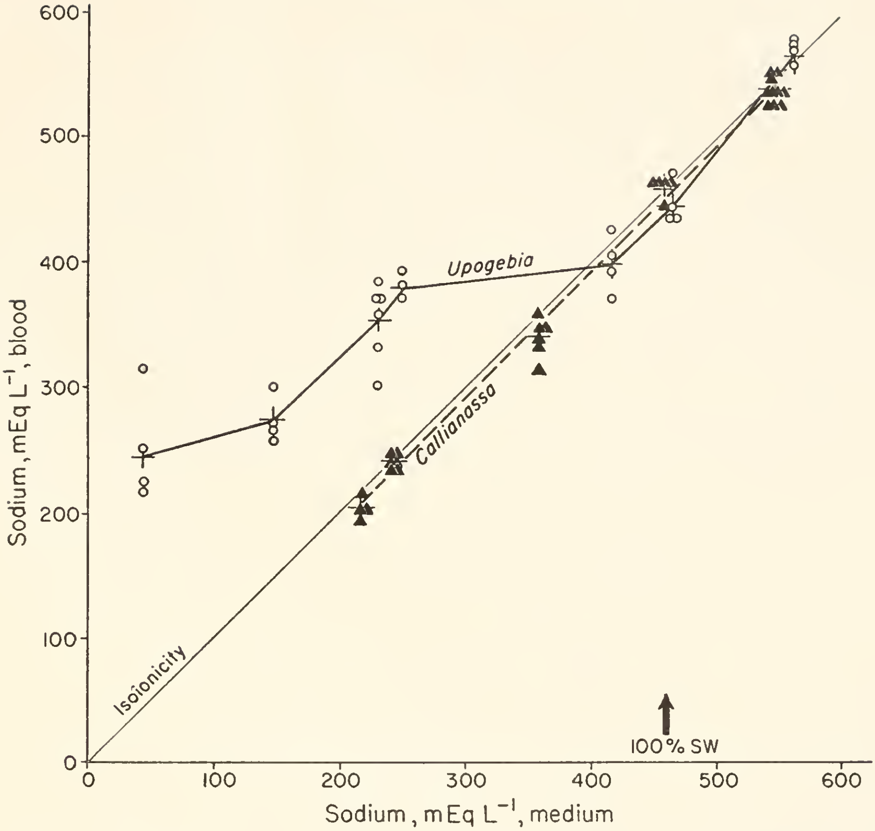

FIGURE 6. Blood sodium of *U. pugettensis* and *C. californiensis* as a function of medium sodium concentration. Temperature of media 15° C. Points represent averages of duplicate determinations. Group means indicated by +.

in 8% SW 7 of 12 animals died within 36 hr. *U. affinis* survived 2 days in 10% SW (n = 4) but 2 animals perished overnight in 5% SW. Three postmolt *U. affinis* survived 10% SW more than 4 days. Under the acclimation regimen, the lower lethal limit for both species of *Upogebia* appears to be *ca.* 10% SW.

C. filholi survived acclimation down to and including 40% SW. Four deaths occurred in 30% SW, 2 in 35% SW and 2 more in 40% SW after holding animals at this latter concentration for 1 week. In preliminary view, between 35%–40% SW appears to be the lower lethal limit for *C. filholi.*

Total osmotic concentration

With respect to blood concentration, *C. californiensis* is an osmo-conformer within its experimental tolerance range (Fig. 5). Where obtainable, urine is iso-osmotic to the blood. Very limited data indicate that the blood of acclimated *C. filholi* is iso-osmotic to the media tested (40%, 60% and 100% SW). Both

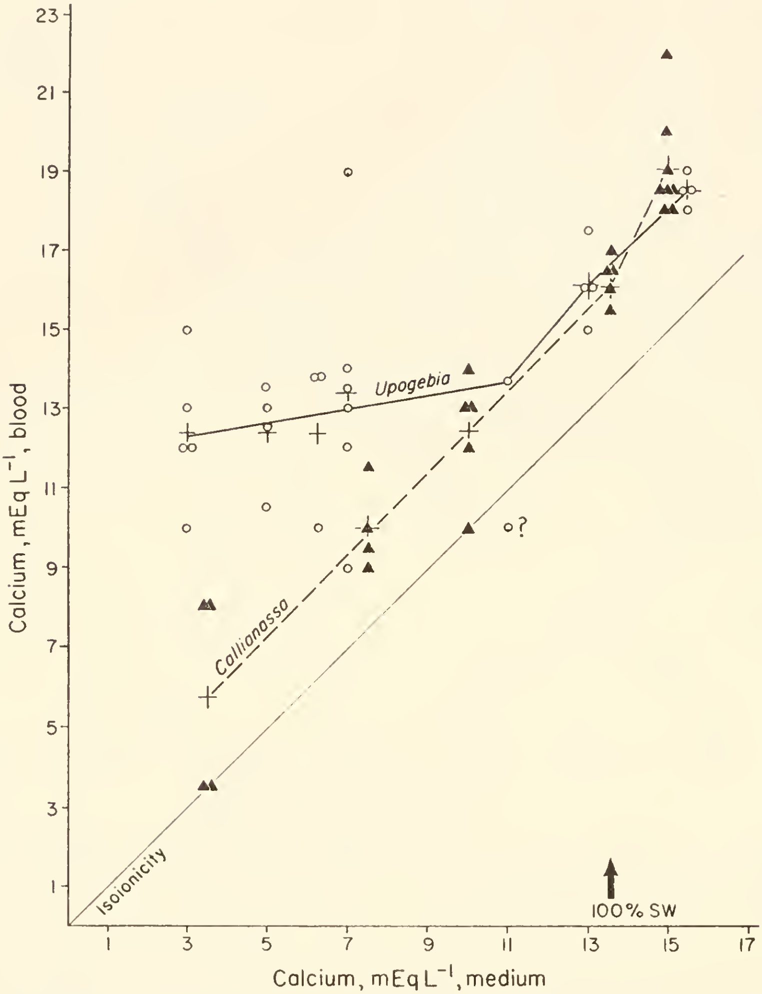

FIGURE 7. Blood calcium of *U. pugettensis* and *C. californiensis* as a function of medium calcium concentration. Temperature of media 15° C. Points represent averages of duplicate determinations. Group means indicated by + with the questioned value omitted.

species of *Upogebia* are iso-osmotic to full strength SW and in *U. pugettensis* this iso-osmoticity extends to 125% SW (Fig. 5). Below 100% SW *U. pugettensis* and *U. affinis* are strong hyper-osmotic regulators. *U. affinis* appears to regulate less well than *U. pugettensis* below 75% SW, although data on the former species are more limited. As in *Callianassa,* the urine of *U. pugettensis* is iso-osmotic to the blood. Data for recently molted *U. pugettensis* are lacking. However, recently molted *U. affinis* survive the range 10%–100% SW for the usual test periods, and measurements indicate that they do so without loss of osmoregulatory ability. As all *U. affinis* molted at least once within a month of their receipt, values presented here probably represent individuals in various stages of the premolt.

Five of 27 *U. affinis* were parasitized in the right branchial chamber by the bopyrid isopod *Pseudione upogebiae*. Values for Cl^- and total osmotic pressure of the blood were essentially the same as for non-parasitized *U. affinis* with the same acclimation history.

Cations

With respect to Na^+ (Fig. 6), *C. californiensis* is iso-ionic, or nearly so, in media down to 50% SW, but *U. pugettensis* commences regulation of this ion at *ca.* 80%–85% SW. *Upogebia* appears to regulate Ca^{++} comparably (Fig. 7) and *Callianassa* is able to maintain a 2–3 meq Ca^{++}/l concentration difference consistently in the media tested. However, there are large variations in the data on Ca^{++} and the findings should be regarded as preliminary, as should the regulatory patterns of blood K^+ (Fig. 8). Both *C. californiensis* and *U. pugettensis* appear to regulate with respect to K^+, but the stronger regulation of *Upogebia* is irregular, showing marked decreases in K^+ levels in the lower salinities.

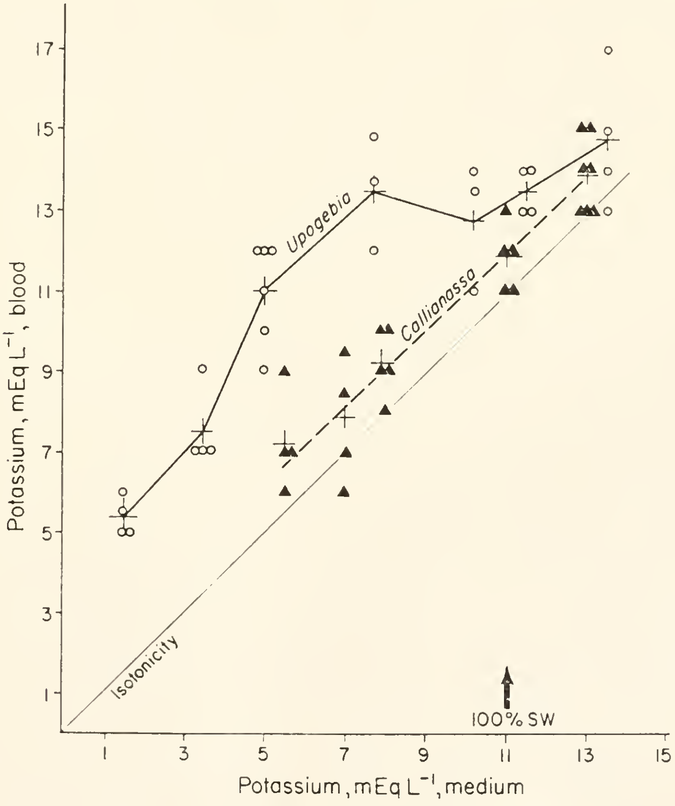

FIGURE 8. Blood potassium of *U. pugettensis* and *C. californiensis* as a function of medium potassium concentration. Temperature of media 15° C. Points are averages of duplicate determinations. Group means indicated by +.

Discussion

Upogebia pugettensis and *U. affinis* resemble certain other characteristically euryhaline Crustacea in their capacity to osmoregulate in dilute brackish conditions. In the higher salinities, both *Upogebia* species conform osmotically, belonging thus to a category of euryhaline crustaceans distinct from those forms which hypo-regulate (Kinne, 1963). Thalassinid urine is iso-osmotic to the blood, a finding not particularly suprising in view of the fact that among decapod hyper-osmotic regulators, distinctly hypotonic urine has been found only in stenohaline FW crayfish (Astacidae), an atyid shrimp (Born, 1968) and palaemonid shrimps (Denne, 1968). Among Peracarida, hypotonic urine has been reported for the amphipods *Gammarus duebeni, G. pulex* and *G. fasciatus* (Lockwood, 1961; Werntz, 1963). The fact that salts are not recovered from thalassinid urine does not imply that the antennary organs of these forms are devoid of osmo-regulatory function. Thus in *U. pugettensis* the antennary organs appear to be necessary for the removal of water whose entry is favored by the maintained osmotic pressure differences and by a relatively high permeability to water (Thompson, unpublished).

Contrary to Pearse's (1945) report, *U. affinis* survives reductions in salinity quite well and this finding is consistent with the regulatory capacities of this species. It seems clear that *U. pugettensis* of Newport, Oregon, is exposed to brackish conditions, and that summer animals, not facing at that time osmotic emergency, retain strong hyper-osmotic regulatory capacity. The same may also be reported for *U. pugettensis* of Bodega Bay, California. It is not known what species of *Upogebia* Gross (1957) utilized in his study. Above Baja California only *U. pugettensis* has been described for the western coast of North America (Stevens, 1928), although questions concerning the taxonomic distinctness of southern California *U. pugettensis* have been raised (MacGinitie and MacGinitie, 1949, pp. 292–3).

The evidence supports the transposition of *Upogebia* from the polystenohaline to the euryhaline category. This view is consistent with the data for *Upogebia littoralis* (Zenkewitch, 1938) and for *U. africana* and *U. capensis* (Hill, 1967). *U. littoralis,* originally found in the Mediterranean Sea (de Man, 1927), is a dominant Black Sea life-form in waters 8–100 m in depth and of an average bottom salinity of 17‰ (Popovici, 1940). *U. littoralis* regulates blood concentration strongly in the range 13%–100% SW. The lower lethal limit was not stated. However, the lower "critical" concentration for both *U. africana* and *U. capensis* is *ca.* 5% SW (Hill, *loc. cit.*). There is limited survival in concentrations less than 5% SW but hyper-osmotic regulation breaks down and death follows molting. Both African species are hyper-osmotic regulators in the lower salinities but become iso-osmotic in the higher SW concentrations.

The present ion analyses of thalassinid blood may be compared to those on the blood of other crustaceans (Duval, 1925). Ion analyses in the iso-osmotic ranges of these thalassinids suggest that strong ionic regulation occurs, as is common in Crustacea (Robertson, 1960). But, for example, the Cl^- hypo-ionicity of *C. californiensis* (Fig. 3) may be attributable to a protein anionic component. Nothing really conclusive on the extent of ionic regulation can be advanced until physico-chemical equilibrium values and transepithelial potentials are measured. Tentatively, therefore, it appears that in *C. californiensis* and

U. pugettensis, Na^+ is at or near equilibrium values, as shown for the eurythaline crab, *Hemigrapsus nudus* (Dehnel and Carefoot, 1965). Over the regulatory range of *U. pugettensis,* the evidence suggests that each ion does not make a relatively constant contribution to the osmotic pressure.

C. californiensis and *C. filholi* cannot osmoregulate yet may be regarded as adapted to survive brackish conditions. Tolerance of dilute media is notable: Animals survive at blood concentrations equivalent to 30% SW or above. Comparatively, the stenohaline marine crabs *Maia squinado* and *Hyas araneus* perish in less than 80% SW and 50% SW, respectively (Duval, 1925; Schlieper, 1929). The anomuran beach crab, *Emerita,* the kelp crab *Pugettia* and some cancroid crabs cannot tolerate less than 75% SW (Gross, 1957). Regarding the whole animal response to osmotic challenge, a second possible factor in survival is delayed time to acclimation. Thus the Cl^- loss rate of *C. californiensis* indicates that, with respect to this ion, a steady state is attained after 18 hr, contrasted to 2 hr for *Emerita* and *C. affinis* (Gross, 1957). A more satisfactory comparison would perforce assess the effects of size and permeability properties upon the rate of equilibration.

An additional factor important for the survival of *Callianassa* is that contacts with the aquatic environment are minimized (*cf.* Kinne, 1967). *C. californiensis* does not possess a well-formed burrow system open at the surface, nor does this species appear dependent upon the integrity of a burrow system for food-gathering (MacGinitie and MacGinitie, 1949) or respiratory functions (R. K. Thompson and Pritchard, in press). The limited data of Table I support the idea that *Callianassa* may be protected from lower interstitial salinities at times of overflowing brackish waters (Reid, 1932) and experience greater salinity stability owing to occupancy of the upper levels of the flats (Milne, 1938).

Since, however, the burrows of *Upogebia* are open to the surface, *Upogebia* is probably more directly exposed to the shallow mudflat waters and consequently to the low and labile salinities of winter and spring. The adaptation of *Upogebia* appears dependent upon the burrow system. Thus the suspension-feeding *Upogebia* (Jørgensen, 1966, p. 88), enclosed in hypoxic or anoxic mud, circulates water through a durable burrow system subserving respiratory and food-gathering functions.

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SUMMARY

1. *Callianassa californiensis* and *Upogebia pugettensis* (Crustacea: Thalassinidea) have been studied with respect to their osmoregulatory capacities and selected aspects of their natural history. *Upogebia affinis* and *Callianassa filholi* have been studied with respect to their osmoregulatory capacities.

2. *Upogebia pugettensis* has a discrete, open and durable burrow system and is distributed further up the Yaquina estuary than is *C. californiensis,* which is without such a burrow system.

3. Resident species of thalassinids are exposed to brackish conditions in the winter and spring at Yaquina Bay.

4. The lower lethal salinity limit for *U. pugettensis* and *U. affinis* is approximately 10% SW; that for *C. californiensis* is 25%–30% SW; that for *C. filholi* is probably 35%–40% SW.

5. *U. pugettensis* and *U. affinis* are strong hyper-osmotic regulators below 75% SW. In full strength SW, *U. pugettensis* and *U. affinis* are iso-osmotic, and the former species conforms osmotically in 125% SW. In *U. pugettensis* the ions of Na^+, K^+, and Ca^{++} are comparably regulated, while Cl^- is probably hypo-regulated in the higher salinities.

6. *Callianassa californiensis* conforms osmotically in the range 30%–125% SW. The ions of Cl^-, Na^+, K^+, and Ca^{++} are relatively poorly regulated. Preliminary findings indicate that *C. filholi* conforms osmotically in the range 40%–100% SW.

7. The urine of *C. californiensis* and *U. pugettensis* is iso-osmotic to the blood.

8. The genera *Callianassa* and *Upogebia* are considered to be euryhaline, but the euryhalinity of the former is more limited.

9. The osmoregulatory capacities of *Upogebia* are considered adaptive to osmotic emergencies which arise, in part, from dependence upon an open burrow system; the osmolability and interstitial habits of *Callianassa* are an alternate adaptive response to its estuarine environment.

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ON THE ORIENTATION OF SEA FANS (GENUS *GORGONIA*)

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Sea fans (*Gorgonia ventalina* and *G. flabellum*) grow in patches at depths of 2 to 10 m on the seaward reefs of the Florida Keys. The sea fans in any particular patch appear to have a preferred orientation of their fan "blades." Théodor and Denizot (1965) have noted this phenomenon and concluded from the parallel orientation of algae and gorgonians that the orientation of all fan-shaped sessile organisms is perpendicular to wave motion and that it may result solely from hydrodynamic phenomena. We have asked the questions, "To what extent do sea fans on Florida reefs show preferred orientation?" and "What can be inferred from the morphology of the sea fan and from the habitat concerning the mechanism that brings about this orientation?"

METHODS

We worked on several coral reef sites on the upper Florida Keys over July and August, 1966, and put in more than 25 man-hours making observations and photographs while using self-contained underwater breathing apparatus. We spent at least one fifth of this time diving through twilight hours and into darkness. We exposed twenty-four 50-foot rolls of Super 8 mm movie film using a Kodak M-6 camera with close-up lenses to record observations and to aid further study of the activities of soft and stony corals.

For measurement of the orientation of sea fan blades to points of the compass, a waterproof compass was mounted in the center of a 12″ square white plastic board. Holding the board horizontally we then placed a side of the board flat against the blade of a sea fan and made a pencil mark on the board at the north arrow. We measured maximum height and breadth of the fan to ± 0.5 cm with a 30 cm rule and recorded these figures next to the orientation mark. We recorded these data for every fan in each patch studied. We surveyed patches in the following places:

1. Carysfort Reef, 3 to 5 m deep, 48 fans.
2. Carysfort Reef, 1 to 2 m deep, 24 fans.
3. Carysfort Reef, 7 to 9 m deep, 49 fans.
4. Key Largo Dry Rocks, 3 to 5 m deep, 26 fans.
5. Conch Reef, 4 m deep, 27 fans.

We computed the mean angular orientation of all fans in each patch and the deviation in degrees of each fan from the mean orientation of its patch. The deviations are plotted against fan height in Figure 1.

After taking the orientation data, we collected several large fans from Conch Reef and cut a transverse slice 2 mm thick from the stem of each fan between the blade and the holdfast. We then traced the outline of the slice and the pattern of concentric growth rings and centers of growth in the axial skeleton with the aid of a camera lucida attachment to a Wild M-5 Stereomicroscope. To each such tracing (Fig. 2) we added a line indicating the plane of the fan at the time it was collected.

Shear modulus of elasticity of the axial skeleton was measured in thick basal pieces and in pieces of the web of the fan. This was done by hanging weights on excised and horizontally supported skeletal fragments and measuring shear displacement at various stresses with a vernier caliper.

RESULTS AND CONCLUSIONS

Figure 1 is a graph showing each fan recorded according to its maximum height (abscissa) and the orientation of its blade with respect to the other fans in its patch (ordinate). All fans from all five patches are shown. If all fans in a patch were perfectly parallel to one another, the points would lie along the abscissa. If the fans were randomly oriented, the points would be randomly distributed over the graph. Reasons for paucity of fans less than 20 cm tall are not clear. Small fans are difficult to find among the abundant plants and animals in that size range. We conclude that the smallest fans are randomly oriented and that there is an increased degree of preferred orientation with increased fan height.

We examined two alternative explanations concerning the mechanism of increasing preferred orientation with increasing size. If fans are oriented to some pattern

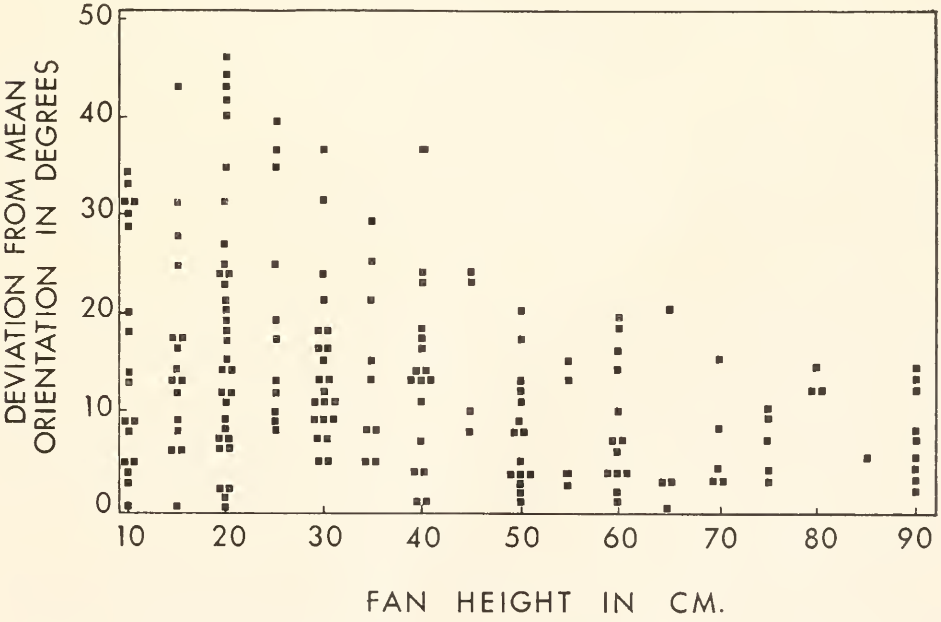

FIGURE 1. Graph showing the height and orientation of all sea fans in five patches on Florida reefs. Orientation is shown as the deviation of each fan's orientation from the mean orientation in the patch. Since the trend illustrated was shown in each of the five patches, data from the five patches are pooled here.

of water movement, are the fans which are not so aligned destroyed by the water movement, thus selecting only the fans that initially had the preferred orientation for each patch? Alternatively, are randomly oriented fans twisted and held perpendicular to the predominant direction of water movement? The nature of the axial skeleton allows it to "give" or display creep under sustained force, and the subsequent addition of axial skeletal material around the older material would tend to hold each fan in its new orientation.

Transverse sections of the axial skeleton of a sea fan show materials of three different textures: (1) axial centers about 0.2 mm in diameter that are not obviously layered and tend to be colorless and transparent; (2) axial medulla, the cola-colored fibrous material (gorgonin) that makes up the bulk of gorgonian axial skeleton, formed into cylinders around axial centers; (3) axial cortex of coarse gorgonin that surrounds the axial medullae in the bases of large fans and that is the mechanically dominant component of the holdfast (Fig. 2).

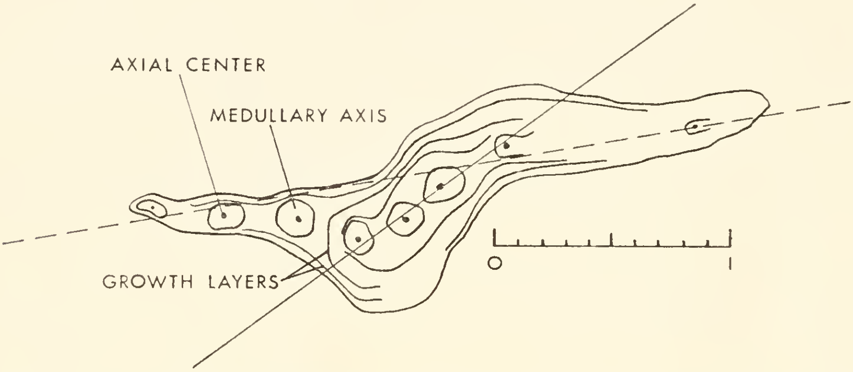

FIGURE 2. Cross-section of the axial skeleton of the stem of a sea fan traced with the aid of a camera lucida. Dashed line: the plane of the fan blade when it was collected. Solid line: possible earlier orientation of fan blade. The number of growth layers indicate the relative age of the axial material: the innermost layers are the oldest. Scale: 1 cm.

The relative age of axial centers can be inferred from the number of layers of axial cortex surrounding each center: the more layers of cortex, the older the center. The alignment of the oldest two or three centers is inferred to be the alignment of the fan at an early stage. The alignment of the outermost (youngest) centers is, we find, the alignment of the fan at the time the fan was collected. In many large fans the early alignment of the blade is seen to be different from the final alignment, indicating a change in orientation of the blade concomitant with growth in height of the blade. We conclude that the orientation of sea fans on shoal Florida reefs can change in time with the growth of the fan.

The fan blade is supported by a few thick axes radiating from the center of the lower edge of the blade and by the even web of axial skeleton of the blade. The axis of the web consists almost entirely of axial center material and has a low shear modulus of 1.22×10^9 dynes cm^{-2} and can undergo extreme lateral deformation: it can be bent double in a distance of 3 to 4 cm without fracturing. The thick basal axis, composed mostly of gorgonin, has a higher elastic modulus, 2.34×10^{10} dynes cm^{-2}, and cannot withstand great deformations. This accounts for the shape taken by fans as they are bent by waves: the basal portion bends very little but the lower modulus, highly deformable upper part is bent to a position parallel to the direction of wave motion. Thus the parts of the fan farthest from the reef surface meet the maximum current velocities (see Discussion, part I) and are the very parts that offer least elastic resistance to the current. Since the current velocity is greater farther from the reef surface, the effect of this orienting force is greater on the larger fans.

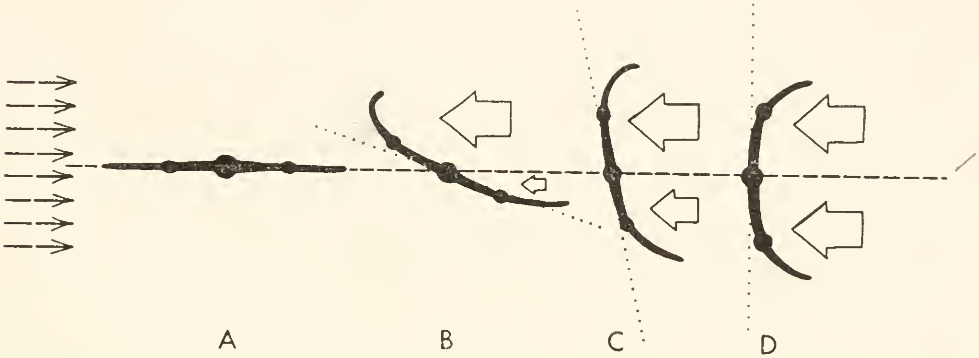

FIGURE 3. Diagram of the orientation of a sea fan (or of any flat, flexible object attached by a stem continuous with a midrib) to the direction of an impinging current as seen from above. Dashed arrows and line: direction of flow. Dotted line: orientation of the fan blade in the absence of current. Outlined arrows: relative magnitude of the forces exerted against the current by leading and trailing edges of the fan blade. A: fan blade parallel (0°) to current and showing no twist. B: fan blade at low angle to the current and showing maximum twist being applied to the stem. C: fan blade at larger angle to the current and showing low twist. D: fan blade perpendicular to the current, also showing no twist.

DISCUSSION

It is highly probable that water movements are the dominant cause of the oriented growth of sea fans. Théodor and Denizot (1965) state that mechanical stability of flexible planar organisms such as sea fans is realized only when the plane of the fan is perpendicular to the direction of impinging water movements. They also state that any twisting of the bases of sessile foliaceous organisms that result from nonperpendicular orientation would decrease their resistance to fracture. By way of partial explanation of why either of these statements should be true, we present the following points of discussion.

I. *Current patterns and fan size orientation.* The velocity of water flowing over a reef varies from zero in the boundary layer within a millimeter of the reef surface to a maximum a meter or so from the reef surface. Coral heads, algae, gorgonians, *etc.*, that project from the reef into the current alter the velocity and direction of flow and create a region of minimum velocity over the reef among these obstacles. These same irregularities will disrupt any laminar tendency in the flow within the region of minimum velocity from that of any prevailing current to a complex turbulent pattern with localized components going in many directions. Small fans growing completely within the region of low velocity could therefore be expected to have random orientation with respect to a prevailing current direction over the reef. As a fan grows out of the turbulent, low-velocity region so that its blade extends into the prevailing current, the current that impinges on these taller fans approaches laminar flow in a predominant direction. These observations are consistent with (1) the observed random orientation of small fans and the preferred orientation of large fans, and (2) the hypothesis that water movements control the orientation of sea fans on the reef.

II. *Current direction and sea fan shape.* The average sea fan is a blade with one or more vertical or nearly vertical, thickened axial supports that extend from the holdfast to the blade and through most of the height of the blade. It is important to note that the blade is almost uniform in thickness and is flexible throughout, while the axial support, being much thicker at the base and tapering from the holdfast to the top of the blade, is stiffer than the blade. A model of this arrangement of blade and axial support can readily be made of plastic. Such a model or a real fan can be moved through water thus simulating flow past a stationary fan while the stem is held by hand.

When the model or a fan is so held and so moved, there are three conditions that are notable (Fig. 3). First, when the blade is exactly parallel (0°) to the direction of flow, and second, when it is perpendicular (90°) to the flow: no twisting moment can be felt in the stem. And third, at any other angle of the blade to the water movement, a twisting moment occurs that is strongest at angles near 0° and decreases as the angle increases to 90°. It is readily seen that at low angles to the direction of flow the leading edge of the fan is bent strongly to one side, thus exposing to the current a surface of almost half the fan (Fig. 3B). This current impinges on the area of the fan perpendicular to the current and produces a large twisting moment. At angles approaching 90°, the trailing edge of the blade presents an increasing area to the impinging current until, at 90°, the forces on both sides of the axial support are equal and there is no twisting moment and hence maximum positional stability.

(1) The shapes that fans take when they are oriented at different angles to the current, (2) the resulting twisting moments and the ability of the fan's supportive system to twist with this moment, and (3) the growth of the axial skeleton by the addition of peripheral layers lead us to suggest that the high degree of preferred orientation observed among large fans in a patch comes about by the fans' being passively twisted and held by the current during periods of growth of the axial skeleton. In this matter, we agree with Théodor and Denizot (1965): we have attempted to explain the phenomenon.

III. *Why sea fans are not parallel to the current.* It remains to explain why, in any patch of fans, one doesn't find half the fans oriented parallel to prevailing currents and the other half oriented at right angles. In a current, small deviations of blade orientation from 90° to the current produce small twisting moments, whereas small deviations from the parallel orientation produce much greater twisting moments. Since sea currents do not hold their directions precisely for long periods of time (see part IV), deviations would be the rule and fans oriented initially parallel to the prevailing current would be subjected to the greatest twisting moments and therefore the greatest tendency to be re-oriented to the stable perpendicular position. We therefore conclude that the direction of preferred orientation of fans in a patch must be perpendicular to the prevailing current.

IV. *Possible stimuli to periodic variations in growth.* The aspect of the subject of sea fans and their habitat that we know least about is the identity and direction of the currents and the time over which they act. *A*. The Gulf Stream sweeps coastal waters northeasterly, parallel to the reef front, during summer months when the prevailing winds are onshore, keeping the Gulf Stream close to the Keys. During winter months prevailing winds are offshore causing the Gulf Stream to move offshore and allowing eddy currents flowing southwest to dominate the water movements over the reefs. These currents are the slowest with the longest period (*ca.* 6 months). *B*. Tidal currents that flow in and out of Florida Bay through the channels between the Keys may provide any directionality according to the position of the reef in question relative to these channels. Close to the channels, the tidal currents will be generally perpendicular to the reef and considerably faster than the currents described in *A*. Their period is a few hours. *C*. Wind-driven surf and surge may come from any direction and have no set period. Direct observations while diving and study of movies show fans on the reef to be constantly waving to and fro with a period of a few seconds. It seems likely to us that some mean direction of all these currents over a full year may be important to the fans. However, we feel that the more violent surf and surge are very likely the most important component in the orientation of sea fans. The short period and alternation of current direction by 180° could provide continuous and constantly renewed directional stimuli to the skeletogenic system of the fan.

Since the axial skeleton of sea fans is a composite material of calcite, fibrous gorgonin containing a collagen-like component (Marks *et al.,* 1949) and probably supplemented by soluble organic matter, it might be expected to share some of the growth-controlling factors with other known composite materials such as bone (Becker *et al.,* 1964) and wood (Kennedy and Farrar, 1965). All these materials have piezoelectric properties and semiconductor properties that can provide electric stimuli to cells in their immediate vicinity. The formative processes of these materials respond to daily, seasonal and annual variations in mechanical stress stimuli by selectively varying the rates of synthesis of the components of the composite material. Experimentally varied mechanical stimuli have been shown to cause variations in growth rates of components in wood (Kennedy and Farrar, 1965) and in insect cuticle (Neville, 1963). Mollusc shells (Barker, 1965) and the skeletons of stony corals (Wells, 1963) show "growth rings" that may represent tidal, daily, lunar, seasonal and annual periods, though experimental evidence for this correlation is lacking. Our own observations of *ca.* 10-μ growth increments in sections 10 to 50 μ thick of axial skeletons of sea fans and other gorgonians lead us to propose a similar mechanism for these organisms: the mechanical stimuli of the waves of surf and surge could be transduced to electrical phenomena by the composite material in the skeleton. These electrical phenomena may then stimulate or inhibit the various synthetic activities of the adjacent skeletogenic cells. The relatively high frequency of this category of water movements renders it subjectively important as a major skeletogenic stimulus.

V. *Mechanical properties of soft vs. stony coral skeletons.* In areas of the sea where water movements have a prevailing direction, many kinds of sessile organisms grow oriented to the prevailing current direction (*e.g.,* Heezen *et al.,* 1966; Riedl, 1966; Théodor and Denizot, 1965; Riedl and Forstner, 1968). Coral reefs abound with sessile organisms and many of them are known to be thus oriented. On Caribbean reefs the species with the most striking degree of preferred orientation is the elkhorn coral, *Acropora palmata,* which has two modes of preferred orientation (Shinn, 1966): one is a flattening of the colony shape that is inversely related to depth and the other is an orientation of branches parallel to prevailing water movements. Since *Acropora palmata* and *Gorgonia flabellum* occur together on many reefs, it is of interest to examine the fact that the sea fan presents its maximum area perpendicular to the prevailing current while the stony elkhorn coral presents its minimum area to the current.

A major function of skeletons is that of support. This is true in plants and animals alike. In general, all solid, nonhydrostatic skeletons can be described as we have in part IV as composite materials (Slayter, 1962). This statement implies that in each supportive structure there are at least two kinds of material: one of high tensile strength and high elastic modulus that lends these properties to the whole structure, and the other a softer, weaker, low modulus material dispersed among units of the first. This softer material serves to dissipate shock stresses concentrated by the more brittle material and imparts viscous properties to the structure.

Solid skeletons can be separated into two categories: those whose largest volume fraction is a mineral (bone, mollusc shell), and those with little or no mineral (wood, insect cuticle, vertebrate tendon). Although the skeletons of *Acropora* and *Gorgonia* have not themselves been analyzed in detail, information on skeletons of related genera is available. The skeleton of stony corals is known to be mainly aragonite and to contain less than 5% by weight of organic matter (Silliman, 1846). In *Pocillopora,* for example, the only insoluble organic material isolated from growing branch tips is chitin in submicroscopic fibrils, oriented randomly within each tangential growth increment (Wainwright, 1963).

The axial skeleton of the gorgonian *Leptogorgia* when dried under tension yields wide angle x-ray diffraction patterns characteristic of collagen (Marks *et al.,* 1949). Sections cut for the present study from the basal axial skeleton of *Gorgonia flabellum* show strong positive axial birefringence throughout most the thickness of each concentric growth increment and a thin, irregularly birefringent component with an axis of preferred orientation which varies around a plane perpendicular to the axis. The small amount of calcite present may or may not be entirely accounted for by the occasional inclusion of mesodermal spicules into the axial substance.

These brief descriptions of the skeletal textures of stony corals and gorgonians can be correlated with what little we know of their mechanical properties. Scleractinian skeleton is rigid and brittle, and any such skeleton found in an area of surf or surge is either massive or encrusting in such a form that compression forces impinging on the coral are effectively withstood. Branches of *Acropora palmata* break cleanly when hit with a hammer. Gorgonians in general and *Gorgonia* in particular are remarkably flexible and exceedingly strong in shear and tension. To remove a large one from the reef, it is easier by far to hack away the reef limestone around the base of it, than to try to break or cut through the basal axial skeleton. Sea fans and other gorgonians can undergo very large deformations in bending, but although they are elastic they are not completely so and unless some restoring force is applied to them, the skeleton will creep and show permanent deformation or at least very long retardation spectra.

Two very different types of supportive systems have evolved that allow both soft and stony corals to grow on surf-beaten coral reefs: (1) Rigid, brittle, highly mineralized stony corals either build massive structures that take waves as compressive forces and if they are branched, they present their smallest projected area to the current. And (2) the supple, highly deformable but visco-elastic, fibrous gorgonians simply bend with the current and, if they are flat, they become effectively oriented to expose their maximum area to the direction of the current. When the current is fast it merely bends them and has not the force either to break their stems or to dislodge their holdfasts from the reef. Since wave action is to and fro by nature, gorgonians are seldom deformed by creep in the skeletal material in response to a unilateral force (Théodor, 1963) but are, in calm moments, seen to stand straight up.

VI. *Trophic adaptations and coral shape.* Laborel (1960) said that the orientation of sessile marine animals is due in part to hydrodynamic factors and is suited to the nutritional mode of each species. Although he was not concerned with hermatypic organisms, he assumed gorgonians fed on particulate organic matter suspended in the sea water. He hypothesized that fan-shaped animal colonies could best collect their food if they were oriented perpendicular to the predominant current. Théodor and Denizot (1965) noticed that all foliaceous animals and algae on horizontal substrata tend to be oriented perpendicularly to wave motion, and since they found no correlation between the incident direction of sunlight and the orientation of foliaceous algae, they discounted the importance of the nutritional mode of any organism in determining its orientation.

We have made concerted efforts to observe and photograph feeding by soft and stony hermatypic corals and to observe the microhabitat around the coral polyps from which they must take nutrient materials. Few gorgonians have been seen on coral reefs actually feeding (Wainwright, 1968), and the known rates of photosynthesis for hermatypic gorgonians are high enough to allow the hypothesis that photosynthesis is their chief mode of nutrition (Kanwisher and Wainwright, 1967). Unpublished observations of feeding activities of stony coral polyps in the field and in the laboratory over ten years and three oceans convince the senior author that the catching of a suspended food particle by a coral polyp of a few millimeters' diameter is not effected simply by erecting the responsive polyp into the main current. We therefore agree with Laborel (1960) in that the hydrodynamic situation at this level is insufficiently known to allow conclusions concerning the relationship of modes of nutrition to patterns of growth and orientation of fan-shaped gorgonians.

Working with pinnately branched hydroids, Riedl and Forstner (1968) have recently reported high correlations among the following: orientation of the colony perpendicular to the direction of current, the bending of the colony, its branches and polyps, and the rate of flow at the point where polyps are actually catching zooplankton. They measured flow rates in the immediate vicinity of the polyps with a minute bead thermistor. Theirs is the first quantitative information we have on the subject, and it is consistent with the hypothesis that orientation of fan-shaped organisms is controlled by hydrodynamic forces. Their information also strongly suggests what we have suspected, namely that the pattern of flow around feeding polyps is under partial control of the size, shape, orientation and physical properties of the colony's support. An extension of this idea is that the polyps' ability to catch zooplankton will depend on these same features of its supportive system.

We wish to express our thanks to John Leikensohn and Jerry Vaughan for diving support and to Drs. Walter A. Starck and Alan Emery for their insightful comments and discussion in the field. To our several colleagues who read the manuscript and to Dr. Sydney Smith who scrutinized and purged the paper of many faults, we are especially grateful.

SUMMARY

1. Measurements were made of the orientation to points of the compass of the plane of 189 sea fans from five patches on shoal reefs in the upper Florida Keys. Small fans were observed to be randomly oriented, whereas large fans showed a high degree of preferred orientation within each patch. Microscopic examination of the axial skeleton of some large fans revealed progressive changes in orientation that had taken place during growth. A passive mechanism of orientation is suggested. Due to the high velocity and short period of surf and surge and the observed motions of fans on the reef, surf and surge are judged to be the most important components of water movements controlling fan orientation.

2. Colonial cnidarians have evolved two different composite systems of effective support against surge on the shoal reefs. (a) Branched stony corals have rigid, highly mineralized skeletons that present their smallest projected area to the current. (b) Alcyonarian (soft) corals have highly deformable, fibrous-organic axial skeletons that expose maximum area to the current.

3. We are just beginning to understand the relationships between biological building materials and the functional supportive systems in which they are found.

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AN AUTORADIOGRAPHIC DEMONSTRATION OF STOMACH TOOTH RENEWAL IN *PHYLLAPLYSIA TAYLORI* DALL, 1900 (GASTROPODA: OPISTHOBRANCHIA)

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Phyllaplysia taylori Dall, 1900, a small, green aplysid (Order Anaspidea, Family Aplysiidae—the "sea hares") common on *Zostera marina* plants along northeastern Pacific shores, has recently been shown to have a diet consisting largely of sessile diatoms (Beeman, 1968). The jaws, radula, and stomach teeth of *P. taylori* are especially well adapted to handling this siliceous, abrasive material. The broad, scraping radula (Fig. 1) removes the food from the substrate and draws it inward between vertical jaws composed of numerous, tightly packed, tiny, hard rods. The esophagus then conveys the food to the triturating stomach or "gizzard," a broad muscular sac (illustrated in McCauley, 1960) lined with corneous intermeshing teeth (Fig. 2). The triturating action here can be so efficient that the identity of food particles in aplysids may not be discernible beyond this point.

Surfaces involved in the grinding of abrasive foods must be repaired or replaced. The rate of replacement for radular teeth, which are replaced as discrete units through slow growth of the radular strap from its posterior origin, has been reported for two pulmonates (Runham, 1963), but nothing is known of such replacement rates in opisthobranchs. The stomach teeth likewise show wear and nothing has been reported of their replacement mechanism or rate. Here my studies using H^3-thymidine autoradiography, although designed primarily to study reproductive mechanisms (Beeman, 1966), have provided pertinent information.

METHODS AND MATERIALS

Specimens of *Phyllaplysia taylori* for this experiment were taken from Elkhorn Slough, California and maintained in special cages in large outdoor seawater tanks (*ca.* 14–16 C) at Hopkins Marine Station. The thymidine used was tritiated at the 5-methyl position and had a specific activity of 2000 mc/mM as supplied by the New England Nuclear Corporation, Boston, Massachusetts.

The experimental series for this study was started on July 25, 1964. The animals used were typically about 7 × 22 mm (*ca.* 190 mg live weight), but they

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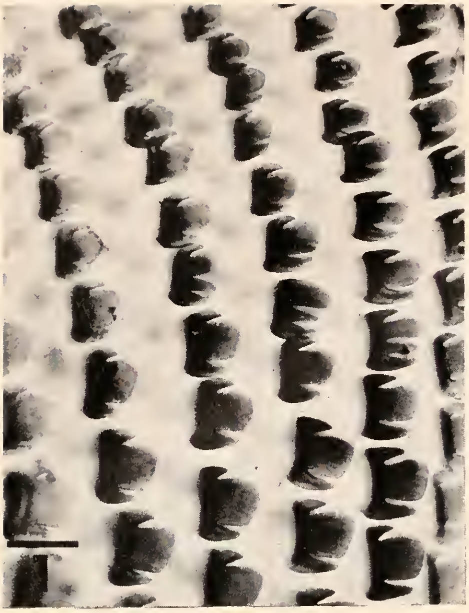

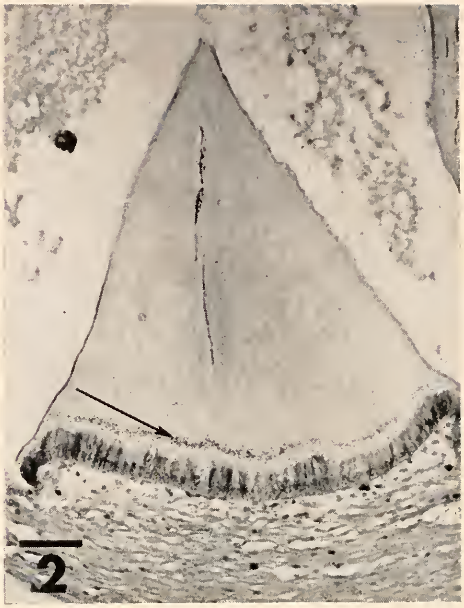

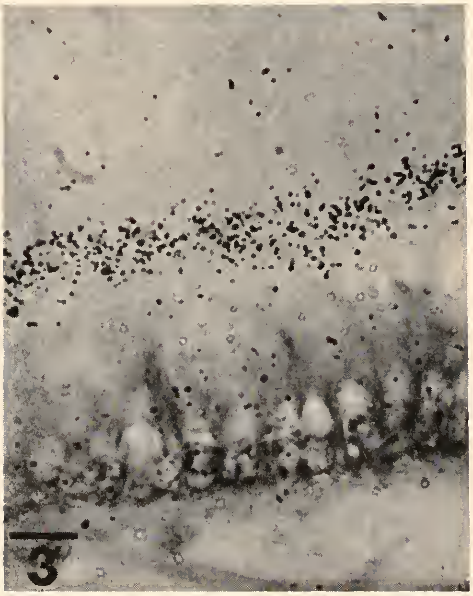

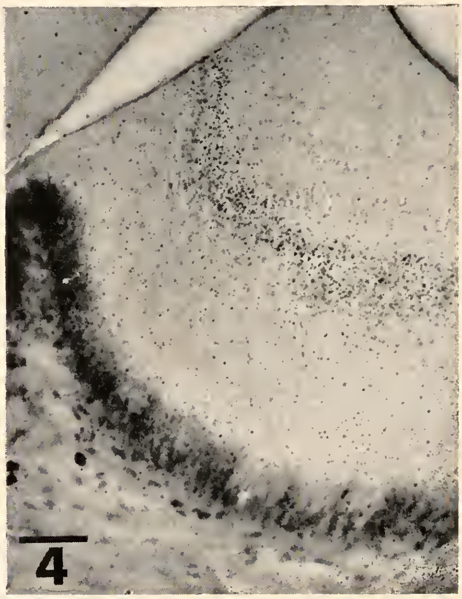

FIGURE 1. Lateral radular teeth of *Phyllaplysia taylori*. Acetocarmine. Scale line represents 50 μ.

FIGURE 2. An autoradiogram showing a sagittal section of a triturating stomach tooth from a *Phyllaplysia taylori* killed 24 hours after injection of tritiated thymidine. Arrow indicates a line of silver grains over the labeled area. Mayer's haemalum. Scale line represents 50 μ.

FIGURE 3. Basal detail of the tooth shown in Figure 2. Focus is in the plane of the silver grains. Scale line represents 10 μ.

ranged from 4 × 18 mm (70 mg) to 9 × 28 mm (536 mg). Experimental individuals were injected with a 31 gage needle into the left anterior quadrant of the hemocoel with 5 μc of H^3-thymidine per gram of body weight and returned to running seawater until sacrificed. Two or three animals were killed by rapid injection of Bouin's seawater solution at each post-injection interval of 1, 2, 4, 10, and 48 hours and 7, 10, 14, 20, 23, and 30 days. Whole fixed specimens, or dissected viscera, were embedded in paraffin, cut at 7 μ, and processed on slides via regular microtechnique. These slides were dipped in Ilford Nuclear Research Emulsion Type K5, exposed in darkness at 5 C for 33, 34, or 78 days, and then developed (six minutes in undiluted Kodak D-19 developer), fixed, rinsed, stained with Kessel's modification of Mayer's haemalum stain for 2 to 15 minutes, "blued" in running tap water for 15–20 minutes, run through an alcohol series, cleared in toluene, and mounted in Permount resin (Joftes, 1963; Holland and Giese, 1965).

RESULTS

Histological preparations which include sagittal sections of the stomach teeth of animals injected with H^3-thymidine show that the teeth have taken up the radioactive label (Figs. 2–4). One hour after injection no label was found associated with the stomach teeth or with their basal cells. Within 10 hours a distinctly labeled band is present at the base of each tooth, just above, but not in the basal cells. Within 24 hours this label line has moved up the tooth, clear of the base, and by 20 days it has almost reached the grinding tip. Total replacement occurs in about 25 days, as no label is present after that post-injection interval. There is no autoradiographic evidence of the migration of labeled nuclei into the growing tooth; the label was found in the non-cellular, translucent matrix of the tooth. Measurement of the position of the labeled band in a total of 17 teeth in five specimens indicates an average growth rate of about 4.9 μ per day. This is a daily replacement of about 4.2% of the mean total height (117.4 μ) of the teeth.

DISCUSSION

The triturating stomach teeth of *P. taylori* are obviously being renewed by secretion of an extracellular matrix from their basal cells. This contrasts with the results of Holland (1965) who autoradiographically demonstrated the incorporation of labeled nuclei into the growing teeth of the sea urchin. There is no evidence of stomach teeth being replaced as discrete units as are radular teeth, but it is conceivable that this could occur on an irregular basis following accidental removal of a secreted tooth.

The main material being secreted into the stomach teeth is probably chitin; the jaws, radula, and stomach teeth of aplysids are usually referred to as chitinous. The positive chitosan color test and the chromatographic demonstration of N-acetylglucosamine, the basic saccharide of chitin, by Winkler (1960) suggest that chitin is present in the stomach teeth of *Aplysia californica* Cooper, 1863, a related aplysid with very similar digestive structures.

FIGURE 4. An autoradiogram of a triturating stomach tooth from a *Phyllaplysia taylori* killed seven days after injection of tritiated thymidine. Mayer's haemalum. Scale line indicates 30 μ.

The nature of the labeling is not clear. The purpose of H^3-thymidine injections is to label newly synthesized DNA. It has been well established (see review by Holland, 1964) that administration of H^3-thymidine causes nuclei to achieve a specific and stable label. However, labeled nuclei are not migrating into the renewed matrix of the stomach tooth. Most firm animal structures are composed of protein or a mucopolysaccharide framework stiffened by additional protein cross-linkages or mineral deposition (Brimacombe and Webber, 1964). The stomach teeth, which at least almost certainly contain chitin, fit this pattern. It is possible that the H^3-thymidine is involved in the formation of the mucopolysaccharide framework. Thymidine diphosphate mannose in some plants and uridine diphosphate glucose, *etc.* in animals are known precursors of mucopolysaccharide. Thymidine might get into the latter compounds as the nucleoside specificity evidently is not as great in saccharide syntheses as in nucleic acid syntheses (Leloir, 1964). However, due to the low stability of the nucleoside bondings to these precursors, the nucleosides would almost certainly stay behind when the synthesized sugars are released from the cell. This would not account for the situation in the stomach teeth, where little or no label is seen to remain in the secreting cells.

There are other ways in which one might account for the presence of H^3-thymidine, or its breakdown products, in the stomach teeth. Holland (1964) has illustrated the steps, in mammals, by which thymidine is degraded to beta-aminoisobutyric acid, which in turn is rapidly excreted in the urine. Although it seems unlikely that beta-aminoisobutyric acid would find its way into the mucopolysaccharide of the tooth, it is not known what other degradation products might occur in mollusks. As Potter (1959) has pointed out, it is not even known how some thymidine breakdown products, which occur when thymidine is incubated with mammalian liver slices, find their way into the Kreb's cycle. Tritiated water, as well as tritiated beta-aminoisobutyric acid, is produced when thymidine tritiated at the 6-hydroxyl group of the pyrimidine ring is degraded (Rubini, Cronkite, Bond and Fliedner, 1960). It is not known if tritiated water occurs when the 5-methyl tritiated thymidine, used in the present experiment, is degraded. Since the degradation products are not known, it is not clear what the non-nuclear label in the stomach teeth may represent. It may be as simple as a labeled acetyl group shunted into the mucopolysaccharide synthesis. Such tritiated acetyl groups would be expected to concentrate in areas of very significant acetylation. The stomach tooth, with its rapid replacement rate, is such a special area. The autoradiographic image resulting from the incorporation of tritiated water would probably be insignificant, due to dilution.

The recent report by Sakai and Kihara (1968) that labeled uridine was unexpectedly incorporated into mouse liver and rat kidney protein supports a suspicion that protein may be bearing the label which appears in the stomach teeth of *P. taylori* after the intrahemocoelic injection of H^3-thymidine. These workers also did not know the mechanisms by which the unexpected labeling occurred, but their work and that of the present study, suggest caution in the interpretation of experimental results, such as autoradiographic images, which follow the *in vivo* administration of labeled thymidine and uridine to higher organisms.

Tooth production and tooth destruction normally must be balanced as I have found only full-size, nicely intermeshing teeth in the functional area of the triturating stomach. Tooth growth probably normally exceeds the minimum need; the shape and size of the teeth being maintained by the milling action of their intermeshing motion. Such a mechanism would account for the addition of well-fitted teeth to the edge of the grinding area and for the enlargement of teeth in growing animals.

Dr. Donald P. Abbott, Hopkins Marine Station of Stanford University, contributed his excellent help at innumerable points. Dr. John H. Philips, also of Hopkins, gave advice on the biochemical aspects of the study and assisted with the critical reading of this paper. Their help, and that of many other associates and family members, is very deeply appreciated. Stanford University and San Francisco State College provided the necessary facilities.

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SUMMARY

1. Brief exposure to H^3-thymidine *in vivo* labels the secretion which basally renews the stomach teeth of *Phyllaplysia taylori.*

2. A line of radioactive label migrates from the base of an average stomach tooth to its tip in about 25 days. This indicates daily replacement of about 4.2% of the mean tooth height.

3. There is no autoradiographic evidence of the migration of labeled nuclei into the growing tooth; the label was found in the non-cellular, translucent matrix of the tooth.

4. The nature of the labeling is not clear. The incorporation of the label into the tooth matrix, probably mainly composed of chitin, may be as simple as the shunting of labeled acetyl groups into the mucopolysaccharide synthesis.

5. Tooth growth evidently normally exceeds the rapid wear caused by an abrasive diet of diatoms. The size and shape of the teeth is maintained by the milling action of their intermeshing motion.

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NITROGEN EXCRETION BY THE SPINY LOBSTER *JASUS EDWARDSI* (HUTTON): THE ROLE OF THE ANTENNAL GLAND

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It is now evident that the antennal gland functions to a greater or lesser extent as an osmotic and ionic regulator in both freshwater and marine Crustacea (Beadle, 1957; Robertson, 1957; Shaw, 1960; Lockwood, 1962; Potts and Parry, 1964). Furthermore, there is a considerable amount of evidence to show that reabsorption, particularly of electrolytes and potential metabolites such as glucose, and secretion may be involved in the process of urine formation as fluid passes through the antennal gland (Martin, 1957, 1958; Parry, 1960; Kirschner, 1967).

Because it produces a urine, is concerned with salt and water regulation, metabolite reabsorption, and has the power to secrete certain molecules, there is a striking resemblance between the functions of the antennal gland of Crustacea and the mammalian kidney. The two organs may therefore be regarded as being physiologically analogous in some respects. However, it would be imprudent to ascribe to the antennal gland all the functions of the mammalian kidney, and to think of it as a true excretory organ, that is, one concerned particularly with the elimination from the animal of nitrogenous waste products of protein metabolism. Whether the antennal gland does play an important part in the excretion of nitrogenous waste products is still in doubt.

Determinations of nitrogen excretion by Crustacea are relatively few. Figures are available for rates of nitrogen excretion by whole animals, without reference to the contribution of urine nitrogen (Dresel and Moyle, 1950; Needham, 1957; Sharma, 1966), or conversely, for urine nitrogenous components with no determinations of their significance to the general problem of nitrogen excretion (Delaunay, 1931). Although it is possible, using the data available, to make a rough estimate of the contribution of urinary nitrogen to overall nitrogen excretion, there has been no specific attempt to relate these two factors directly. This omission is perhaps surprising, and represents a real gap in our knowledge of antennal gland function in Crustacea.

The aim of this study was to determine the role of the antennal gland of *Jasus edwardsi* in relation to general nitrogen excretion by this animal. Nitrogen loss from whole animals was measured and the contribution of urinary nitrogen to total nitrogen excretion was estimated by determining various nitrogenous components in the urine and the rate of urine flow. In this way it was intended to define, more accurately than current information allows, the relative importance of the antennal gland as an excretory organ.

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MATERIALS AND METHODS

Animals were collected at Kaikoura, on the North Canterbury coast, and transported immediately to the laboratory in Christchurch. They were kept in a large concrete tank supplied with aerated, circulating sea water maintained at a temperature of 14 ± 0.5° C. Animals were used within a week of being collected, and were not fed while in the holding tank or during experiments.

Total nitrogen (TN) and ammonia nitrogen (AN) excretion were determined by analyzing small volumes of sea water in which an animal had been living. The experimental animal, after being dried as thoroughly as possible, was weighed and then left overnight in a large glass desiccator containing 1500–2000 ml of filtered sea water (Millepore, mean pore diameter 0.45 μ). The desiccator lid, sealed with Vaseline and clamped in place, was closed at the top with a rubber bung which contained an air inlet into the sea water and also a shorter outlet.

Jasus was found to be extremely sensitive to oxygen lack, and continuous aeration of the sea water was necessary. A current of air was passed first through a large boiling tube containing 1 N sulfuric acid to remove any ammonia, then through a second tube containing distilled water before passing into the sea water in the experimental chamber. Air leaving the chamber passed through two further tubes, each containing 50 ml of 0.01 N hydrochloric acid.

AN in the bathing medium was determined by direct steam distillation of 10 ml samples of the sea water in a Markham unit, after the addition of 10 N sodium hydroxide solution, and subsequent titration of the distillate against 0.01 N hydrochloric acid. TN was determined by the Kjeldahl method. Any ammonia which may have passed with the current of air from the bathing medium was carried over into the two tubes containing dilute acid. This nitrogen fraction, determined by titration of the acid against a dilute standard solution of sodium hydroxide, was taken into account when rates of excretion of TN and AN were calculated.

Urine samples were obtained as follows. The areas around the nephropores were dried, and the anterior openings of the gill chambers blocked with small wads of filter paper. The membrane covering the openings from the bladder appeared to be extremely irritable; slight pressure on the membrane with the tip of a glass micropipette was usually sufficient to produce a flow of urine which was then drawn into the pipette by suction. In some cases it was necessary to push the tip of the pipette through the opening in the membrane to obtain a urine sample. This was done with extreme care to prevent any tearing of tissues, and urine obviously contaminated with blood or tissue fragments was discarded.

The total nitrogen content of urine was determined by the Kjeldahl method. Ammonia and urea in urine were determined by the microdiffusion method of Conway (1950), urea being estimated after treatment of aliquots of urine with powdered urease in an acetate buffer. Amino compounds in the urine were determined by the method of Rosen (1957), using a Bausch and Lomb Spectronic 20 colorimeter. Concentrations of amino compounds were related to glycine standards which were used throughout.

To estimate quantitatively the contribution of urinary nitrogen to general nitrogen excretion, it was necessary to determine urine production rates of animals. The openings from both antennal glands were blocked with dental cement. Animals were then weighed, placed in sea water and re-weighed after a period of 6 to 8 hours. Rates of urine flow were calculated on the assumption that weight increase was due to the accumulation in the bladder of urine which would normally have been voided.

Duplicate analyses were made for all determinations of the various nitrogen components in both bathing medium and urine samples.

The composition of body fluids and the rate of urine flow of some Crustacea may be affected by experimental stress or excessive handling (Riegel, 1960; Riegel and Kirschner, 1960). In the present study, all chemical analyses and determinations of urine flow rates were made using animals which had not previously been used for experiments. Animals could therefore be regarded as being as near 'normal' as possible when measurements were made.

RESULTS

The rates of excretion of TN and AN into the surrounding medium, and AN as a percentage of TN loss are shown in Table I.

TABLE I

Rates of excretion of total nitrogen and ammonia nitrogen by Jasus edwardsi

Animal no.	Total nitrogen (TN) (μg N/hr/g body wt)	Ammonia nitrogen (AN) (μg N/hr/g body wt)	$\frac{AN}{TN} \times 100$
1	4.0	3.2	80.0
2	2.1	1.8	85.7
3	3.8	2.7	71.1
4	4.1	2.7	65.8
5	1.7	1.2	70.6
6	1.0	0.6	60.0
7	8.8	—	—
Mean ± S.D.	3.6 ± 2.4	2.0 ± 0.9	72.2 ± 8.5

TN excretion rates varied over a considerable range, the fastest rate being almost nine times greater than the slowest rate recorded. However, at all rates of excretion AN represented a consistently high percentage of TN loss, with a mean value of 72.2% TN and a range of 60.0% to 85.7%. It is considered that this figure represents as accurate an estimate of AN as possible under the conditions of these experiments. Bacterial decomposition of nitrogenous components other than ammonia in the bathing medium could conceivably increase the recorded AN/TN ratio above its true value. Because of this possibility, sea water was initially made bacteria-free by filtration and animals were kept in the experimental chamber for only a short time, to ensure that changes in the ammonia content of the sea water due to the action of bacteria were minimal.

Concentrations of the four nitrogenous urine constituents measured are shown in Table II.

Of the three specific components determined, urea represented the largest fraction. However, urea, ammonia and amino compounds together represented only 21.2% of total urine nitrogen. By far the greatest proportion of urine nitrogen was due to constituents other than these three common excretory products.

TABLE II

Concentration of total nitrogen, ammonia, urea and amino compounds in the urine of Jasus edwardsi

	Urine nitrogenous constituents				
	Total urine nitrogen	Ammonia	Urea	Amino compounds	Unidentified (by substraction)
Mean conc. (mg. %)	21.2	1.5	4.7	5.7*	
S.D.	±4.1	±0.7	±1.8	±3.1	
Mean conc. (μg N/ml)	212	12.6	21.8	10.6	167
% total urine nitrogen	—	5.9	10.3	5.0	78.8
No. of observations	7	7	6	6	

* Corrected for urine ammonia and expressed as equivalent to glycine concentration.

The determination of urine flow rates in aquatic animals is a difficult problem, and the method used in this study is open to various criticisms. Primary urine is probably produced by ultrafiltration of the blood into the antennal gland of Crustacea (Kirschner, 1967). In blockage experiments it is possible that the accumulation of large volumes of urine in the bladder could produce a back pressure sufficient to reduce the rate of urine flow. For this reason, animals were re-weighed a relatively short time after the urinary openings were blocked, so that the flow of urine through the antennal gland itself was unimpeded.

Rates of urine production in Table III are remarkably consistent, and it is unlikely that this degree of consistency would have been achieved if the weight increases observed were due to factors other than the accumulation of urine, such as the swallowing of sea water by experimental animals.

The assessment of the contribution of the nitrogenous constituents in the urine of *Jasus* to overall nitrogen excretion by the animal is shown in Table IV. The percentage contribution of urine nitrogen fractions to TN and AN excretion was assessed using the mean values for nitrogenous excretion rates, urine constituents and flow rates. In calculating the volume of urine produced per unit time it was

TABLE III

Rates of urine production in Jasus edwardsi

Animal no.	Body weight (g)	Urine production rate (% body weight/day)
15	166.9	4.3
16	165.8	3.1
17	198.3	6.5
18	178.3	3.8
19	122.8	4.8
20	244.6	6.2
	Mean	4.8
	S.D.	±1.1

TABLE IV

Contribution of nitrogenous constituents in the urine of Jasus edwardsi *to total nitrogen and ammonia nitrogen excreted*

Urine nitrogenous constituent	% contribution to total nitrogen excreted	% contribution to total ammonia excreted
Ammonia	0.7	1.3
Urea	1.2	—
Amino compounds	0.6	—
Unidentified	9.1	—
Total nitrogen	11.6	—

assumed that the urine had the same specific gravity as the sea water used in experiments which, at room temperature, was 1.022.

Nitrogen loss in the urine would account for 11.6% of TN excreted. However, the contribution of the common excretory products ammonia, urea and amino nitrogen would be only 2.5% of TN excreted. The large unidentified nitrogen fraction, presumably itself made up of several components, would represent 9.1% of TN excreted. The main excretory product of *Jasus* is ammonia (Table I), yet urine ammonia would contribute less than 1% of TN loss, and only 1.3% of AN excreted by the animal.

DISCUSSION

The general pattern of nitrogen excretion in *Jasus* is typical of that shown by a large variety of aquatic invertebrates (Prosser and Brown, 1961), in that the principal excretory product of the animal is ammonia. This compound represented just over 70% of TN excreted by *Jasus*, and equally high AN/TN ratios have been recorded for other aquatic Crustacea (for review see Parry, 1960). In addition to this qualitative similarity, it is clear from Table V that the amount of nitrogen excreted by *Jasus* lies within the range of excretion rates per unit body weight measured for other aquatic Crustacea.

TABLE V

Nitrogen excretion rates in some aquatic crustaceans

Species	Rates of nitrogen excretion (mg N/10 g body wt/day)	Reference
	Total nitrogen	
Jasus edwardsi	0.9	This paper
Carcinus maenas	0.4	Needham (1957)
Gammarus locusta	4.9	Dresel & Moyle (1950)
G. zaddachi	6.0	Dresel & Moyle (1950)
G. pulex	2.3	Dresel & Moyle (1950)
Marinogammarus marinus	1.1	Dresel & Moyle (1950)
M. pirloti	2.9	Dresel & Moyle (1950)
	Ammonia and urea only	
Orconectes rusticus	3.5	Sharma (1966)

Although there appears to be some variation in the rates of nitrogen excretion between different species, some standardization of experimental procedures will be necessary before it can be established whether or not these variations indicate real differences in excretion rates. Rates of nitrogen excretion for a single species may vary over a considerable range depending on such factors as the nutritional state of animals, the presence or absence of other animals, stage in molt cycle or the volume of sea water in which an animal is living (Needham, 1956, 1957).

Delaunay (1931) found that the concentrations of ammonia, urea, uric acid and amino nitrogen in the urine of *Maja* were extremely low, and concluded that in this animal the antennal gland was not primarily concerned with nitrogen excretion. Similarly, Parry (1960) reviewed work on antennal gland function in Crustacea, and considered that the information available showed conclusively only that the antennal gland of marine Crustacea was important as an ionic regulator, and that its role in general nitrogen excretion was likely to be of little consequence.

The present study is the first direct attempt to quantify the role of the antennal gland in relation to total nitrogen excretion. The common excretory products measured in the urine would contribute an extremely low proportion of TN and AN excreted by *Jasus*. Almost 90% of all soluble nitrogen excreted is non-urinary in origin. The waste nitrogen is mainly in the form of ammonia, and presumably much of it leaves the animal by diffusion through highly permeable surfaces such as the gills. Therefore, in terms of overall nitrogen loss from *Jasus*, urine nitrogen is of very minor importance.

Despite this conclusion, the antennal gland may be concerned with the excretion of materials which cannot be disposed of by simple diffusion. Most of the nitrogen in the urine of *Jasus* was not identified, and this unknown fraction would contribute almost 10% of TN excreted by the animal. Equally large amounts of unidentified nitrogen in the urine of other Crustacea are simply a reflection of the very limited range of analyses which have been carried out on urine samples. Secretory activity is widespread in various parts of the antennal glands of Crustacea (Lison, 1942). Ramsay (1961) suggested that secretion may be of importance in eliminating complex nitrogenous compounds which are either too big or otherwise unsuitable to enter the antennal gland by filtration, and that secreted materials could account for at least some of the nitrogen hitherto unidentified in the urine of Crustacea.

Secretion/digestion systems of the type known to be present in the antennal gland of the freshwater crayfish (Riegel, 1966) are likely to be present in the antennal glands of other Crustacea. Such systems may be important in the elimination of complex waste products, and further detailed analysis of the urine of Crustacea may reveal that the antennal gland does have a true excretory function, although it appears that the organ does not have a quantitatively important role in overall nitrogen excretion.

SUMMARY

1. Nitrogen excretion by *Jasus edwardsi* has been investigated.

2. Ammonia nitrogen accounted for 72% of total nitrogen excreted.

3. Urine production rate was 4.8% of the body weight per day, and urine nitrogen would contribute 11.6% of the total nitrogen loss from the animal.

4. Ammonia, urea and amino compounds represented 21.2% of total urine nitrogen. These compounds together accounted for 2.5% of total nitrogen excreted, and urine ammonia for 1.3% of total ammonia loss.

5. Most nitrogen excreted is non-urinary in origin, and it is concluded that the antennal gland of *Jasus* is not important as far as total nitrogen loss from the animal is concerned.

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THE SURVIVAL OF OSMOTIC STRESS BY *SYPHAROCHITON PELLISERPENTIS* (MOLLUSCA: POLYPLACOPHORA)

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Osmotic studies on invertebrates may be broadly divided into those on fresh- and brackish-water species, which generally show some degree of osmotic regulation; and those on marine species, which are typically isosmotic with seawater. Many littoral marine animals are however exposed to osmotic changes, and have evolved various mechanical and physiological methods of surviving these stresses. There seem to be no published data on osmotic studies with chitons (a wholly marine group), except a note by Arey and Crozier (1919) on the sensitivity of parts of the body of *Chiton tuberculatus* to osmotic stimulation. Indeed Robertson (1964, p. 284) states, "complete gaps exist for whole classes such as the Amphineura and Scaphopoda."

The subject of this investigation is the common New Zealand chiton *Sypharochiton pelliserpentis* (Quoy and Gaimard, 1835). This is the name at present in general use (Knox, 1963; Morton and Miller, 1967), but there is unpublished work (Johns, 1960) suggesting that the genus *Sypharochiton* (Thiele, 1893) should be relegated to *Chiton* (Linnaeus, 1758). *S. pelliserpentis* is a very common littoral animal on rocky shores throughout New Zealand. It penetrates harbors and estuaries to a certain extent, though this does not bring it into contact with seawater more dilute than about 30‰S. It occupies a broad vertical range on the shore, from open rock to small water-filled hollows, depressions and crevices. These small bodies of water have been found by the present author to be subject to salinity fluctuations of at least 14‰ to 45‰S over very short time periods. The chitons may be exposed to severe osmotic stress for periods of up to 10 hours. The problem, then, is how *S. pelliserpentis* survives these fluctuations in the probable absence of any efficient osmoregulatory ability.

Chitons normally live permanently attached to hard substrates, detachment being the result only of some external force. Unlike some species, *S. pelliserpentis* is unable to right itself when detached and placed upside down on a flat surface. For these reasons the experiments reported here were carried out on animals firmly attached to glass substrates, but some comparative weight change experiments were performed on unattached animals. The salinity conditions used spanned the extreme range recorded in the field, and experiments continued for 24 hours, to exceed the exposure time naturally possible.

METHODS

To minimize possible intraspecific adaptive differences, adult animals were always removed from one beach at the same vertical level and from the same substrate. They were kept in the laboratory in seawater collected from the site at the same time. The salinity of this was determined, with an inductive salinometer or silver nitrate titration, and fell within the range 34.2‰ to 35.7‰S. Hypotonic media were made up by diluting this 100% seawater with the buffer $M/400$ $NaHCO_3$; "0% seawater" was a solution of $M/400$ $NaHCO_3$ in distilled water. Concentrated seawater (150%) was prepared by evaporation. The animals were used within a few days of collection, the experiments being carried out in 4 liters of experimental solution, contained in "Perspex" tanks and continuously aerated with porous stone diffusers. The tanks were covered with "Perspex" lids to reduce evaporation and consequent salinity change.

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Survival of the chitons was measured on the basis of recovery after a period in a favorable environment. At the end of each experiment, animals were transferred at once to a tank containing full strength aerated seawater. After 24 hours in this, the recovery medium, the animals were removed, placed on their dorsal surfaces and scored as follows. Animals which were attached at the end of the recovery period, and in which contractile waves began to pass along the foot shortly after being placed on their dorsal surfaces, and which reattached readily, were designated fully alive and scored 2. Those which were unattached at the end of the recovery period, and immobile when placed on their dorsal surface, but which reacted by feeble curling to light mechanical stimulation of the head, foot or gills, were designated moribund and scored 1. Animals unattached at the end of the recovery peroiod, immobile when on their dorsal surfaces, and unreactive to mechanical stimulation, were designated dead and scored 0.

Using this scheme there was little difficulty in deciding the death of any particular animal. The total score for recovered animals was then expressed as a percentage of the possible total had all animals in the group recovered fully.

Animals were weighed in air in groups attached to glass plates, or individually when unattached. After removal from the experimental medium they were blotted with a soft dry towel and weighed together with the glass plate. Unattached animals were similarly removed and blotted, then weighed individually in a polythene sling from the arm of a torsion balance. The weights in every case were expressed as percentages of the initial weight.

Measurements of the freezing-point depression were made on samples of fluid withdrawn from the pericardial cavity. The total osmotic pressure on the pericardial fluid was assumed to be the same as blood (Potts and Parry, 1964). This has been shown to be so for some lamellibranchs and gastropods (Picken, 1937), though more recent work on the fresh-water snail *Viviparus viviparus* showed slight differences in the O.P. of blood and pericardial fluid (Little, 1965). Indeed this would be expected if pericardial fluid is a filtrate of the blood (Harrison, 1962). No differences in the O.P. of the blood and pericardial fluid of three species of littorinid winkles could be found by Todd (1964). In the chiton, with its sluggish circulation (Martin, Harrison, Huston and Stewart, 1958) and probably very low non-electrolyte content of the blood, the assumed isosmoticity of pericardial fluid and blood seemed justified in view of the gross osmotic changes to be investigated here.

The pericardium lies directly beneath shell valves VII and VIII, and fluid was removed directly by piercing the dorsal body wall between these two valves and inserting a fine "Pyrex" glass capillary tube. The sampling tube was first filled with liquid paraffin, a short column of sample drawn in, followed by more liquid paraffin. In this way duplicate or triplicate sub-samples (0.01 μl to 0.1 μl in volume) were removed from the animal and drawn up the tube between liquid paraffin columns. Difficulty in removing fluid samples was only experienced with animals after prolonged exposure to 150% seawater. Samples visibly contaminated with cell debris or genital products after accidental rupture of the gonad were rejected immediately. The tubes were placed at once on solid carbon dioxide, and could be stored at −18° C without measurable change in melting-point of the sample. Animals suffered irreparable damage and could only be sampled on one occasion.

The freezing-point depressions (Δ° C) of these samples was determined by allowing them to warm up slowly in a continuously stirred alcohol bath. The frozen samples were viewed with a binocular microscope through crossed "Polaroids". The temperature at which each sample melted was read on a chemical Beckmann thermometer, graduated in 0.01° C divisions, and compared directly with that for distilled water sampled in a same way and included in the bath for each experimental run. The Δ° C of the seawater media of the animals was similarly determined for each experiment.

Temperature of experiments was controlled by suspending the "Perspex" tanks in a large constant temperature bath. A low temperature of 10° C was used, approximately the lower limit of sea surface temperatures in Auckland (Skerman, 1958). At the upper end of the scale, a temperature of 30° C was used, this temperature being frequently attained or exceeded in small littoral pools during summer. Room temperature during the experiments was 20° C ± 2° C.

Results

Survival and weight changes in various salinities

The percentage survival of attached *Sypharochiton pelliserpentis* (groups of 10), in 0%, 50%, 100% and 150% seawaters, after 2, 6, 10 and 24 hours exposure was determined. The experiments were repeated at three temperatures, 10° C ± 0.1° C, 20° C ± 0.5° C and 30° C ± 0.1° C. The data for survival at a controlled temperature of 20° C are taken to indicate survival at room temperature. Survival was 100% after 24 hours in concentrated (150%) or normal (100%) seawaters at 20° C and 30° C, but between 95% and 100% at 10° C. In 50% seawater, survival was 80 to 85% after 10 and 24 hours exposure at 10° C, but was 100% at 20° C. At 30° C, survival after 10 hours was 85%, but after 24 hours was only 5%. Freshwater (0% seawater) resulted in 85% survival after 10 hours and 15% survival after 24 hours at 10° C. At 20° C in 0% seawater, mortality occurred between 10 and 24 hours exposure, there being only 10% survival after this time. In 0% seawater at 30° C, survival was down to 80% after only 2 hours, and there were no survivors after 6 hours in this medium at this temperature.

Summarizing these results, under laboratory conditions *S. pelliserpentis* survived well in 150% and 100% seawaters for 24 hours at temperatures of 10° C to 30° C. Half strength (50%) seawater caused significant mortality (greater than 50%) only at 30° C and after exposures longer than 10 hours. Freshwater (0%

seawater) caused significant mortality only after 10 hours at 10° C and 20° C, but after 2 hours at 30° C.

Mean weight changes of groups of attached *S. pelliserpentis* in several experimental seawaters are shown in Figure 1 (each point is the mean of 5 animals). A rapid increase in weight resulted from transfer to hypotonic media. In 75% seawater weight equilibrium was reached and followed by a decrease towards the initial weight. Animals in 50% seawater reached equilibrium more slowly and showed a small decrease towards the initial weight. When transferred to more dilute media (25% and 0% seawaters) animals did not attain weight equilibrium within 24 hours.

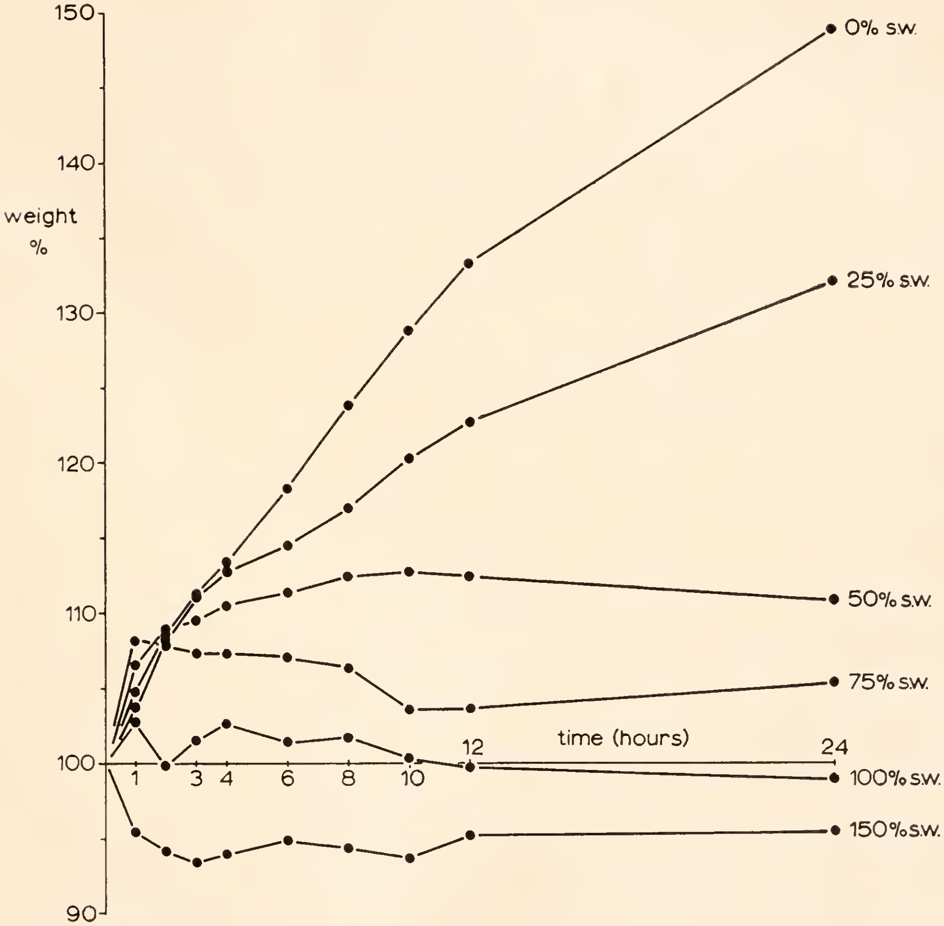

FIGURE 1. Mean weight changes (as % of initial weight) of groups of attached *S. pelliserpentis* in several experimental seawaters. Room temperature.

Control animals, remaining in the stock 100% seawater but weighed at the same intervals as the experimental animals, showed small weight fluctuations about the initial weight. In hypertonic seawater (150%) a small weight loss was recorded, not proportional to the weight increase in 50% seawater.

These experiments were repeated with unattached animals and the results are shown in Figure 2. Here the initial weight increase in hypotonic media was more rapid. Survival of these animals after recovery was identical with that of the attached animals, except that after exposure to 25% seawater, survival was only 80%. No significance is attached to this difference.

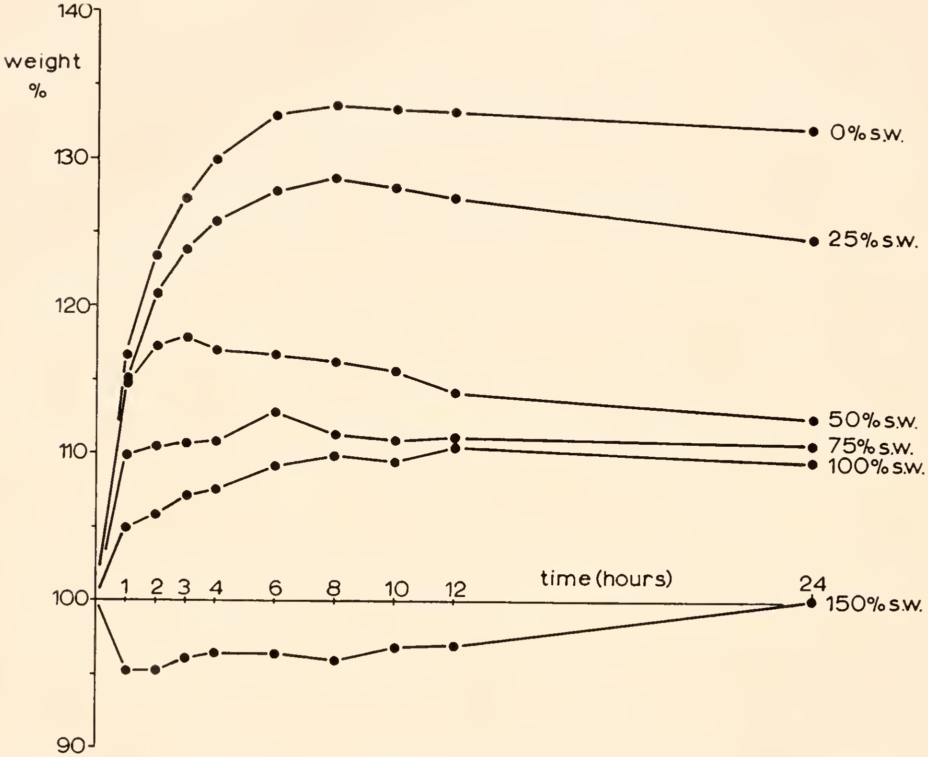

Figure 2. Mean weight changes (as % of initial weight) of unattached *S. pelliserpentis* in several experimental seawaters. Room temperature.

Unattached control animals consistently showed a steady weight increase when no osmotic gradient was present. An interpretation of this is based on the animals' behavior. The initial weight was derived from animals just detached from the tank wall and blotted while extended. Following this the animals tended to curl while in air. On return to the experimental tank some extended and actively flexed backwards, others remained partly curled. As the experiment progressed and after further disturbances for weighing, all animals showed an increasing tendency to remain curled. The gradual weight increase shown in Figure 2 could be due to this progressive curling tendency, trapping water in the mantle groove. Attached chitons in the same control situation often showed slight fluctuations, but never as great or as consistent as those of unattached animals.

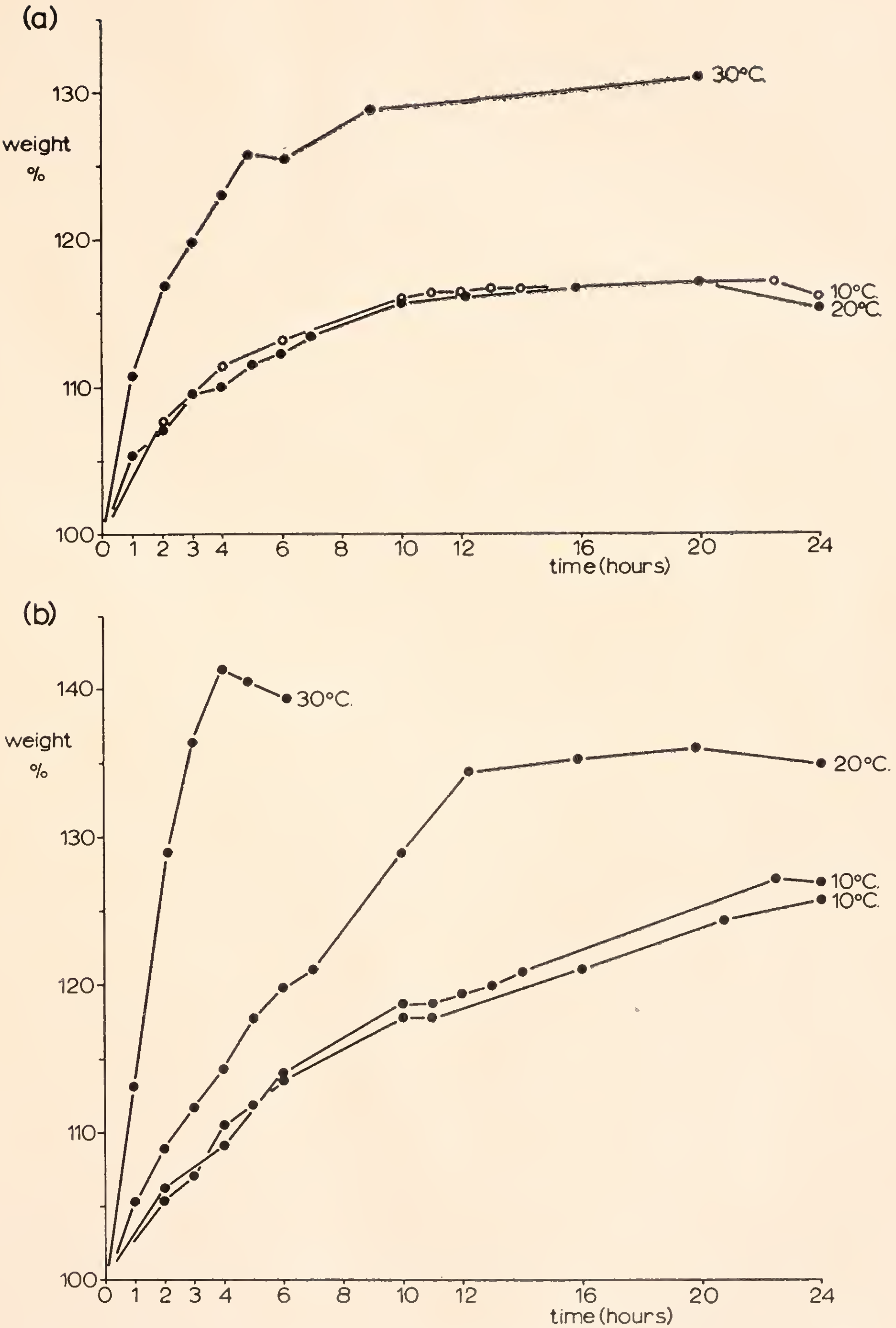

FIGURE 3. Effect of temperature on weight changes of groups of attached *S. pelliserpentis* in (a) 50% seawater, and (b) 0% seawater.

Hypertonic seawater (150%) caused a small weight loss, not proportional to the weight gain in 50% seawater. After 24 hours in this medium, animals almost regained their initial weight.

The results of repeating these weight change experiments on attached animals in hypotonic media at three controlled temperatures, are plotted in Figure 3. At 20° C (Fig. 3b), apparent weight equilibrium in 0% seawater was reached, contrasting with the absence of weight equilibrium in similar conditions in the experiment shown in Figure 1. At this stage, considerable mortality would have resulted from the experiment.

Osmotic adjustment in various salinities

Experiments on the relation of the internal and external osmotic pressures in hypotonic seawaters are summarized in Figure 4. Each point is the mean value for Δ° C of 8 to 10 animals, and the time after transfer to hypotonic conditions is shown on each curve.

Clearly the osmotic gradient between the animal and its surrounding medium decreases rapidly with time. Adjustment takes place. Increase in temperature accelerates the rate of adjustment.

It is shown in Figure 5 that pericardial fluid is normally isosmotic with seawater between 10° C and 30° C. In 150% seawater the internal concentration adjusts very rapidly, usually within two hours of transfer.

Behavioral reaction to salinity change

Unattached animals remained curled after repeated weighings; this took place regardless of salinity and was assumed to be a result of disturbance. It has been discussed relative to weight changes of control groups of animals.

With attached animals, two aspects of behavior were considered; the initial movement of the animals after transfer to the medium, and the length of time for which movement continued. Normally, when animals attached to glass plates were immersed in 100% seawater, they began at once to move about and crawl over one another. Continuous movement soon stopped but intermittent activity occurred over 24 hours. If repeated weighings of them were made, the same pattern of movement was repeated but the duration of continuous movement decreased after each return to the water.

In 75% seawater movement was almost normal, the animals showing some activity for most of a 24-hour period. When transferred direct to 50% seawater however, animals showed more vigorous initial movements, raising and lowering the girdle, turning to and fro, and raising the head. They remained active in this medium for an hour or two, finally attaching firmly in one place. Most were still attached in the same place after 24 hours.

In 0% seawater the initial movements were greatly exaggerated, animals immediately moving, raising the girdle, and flexing backwards exposing the anterior ventral surface. This continued for a few minutes only, after which they attached very firmly, not to move again until the termination of the experiment. After 24 hours in this medium the animals were frequently detached—forced off the substrate by the swelling of the foot.

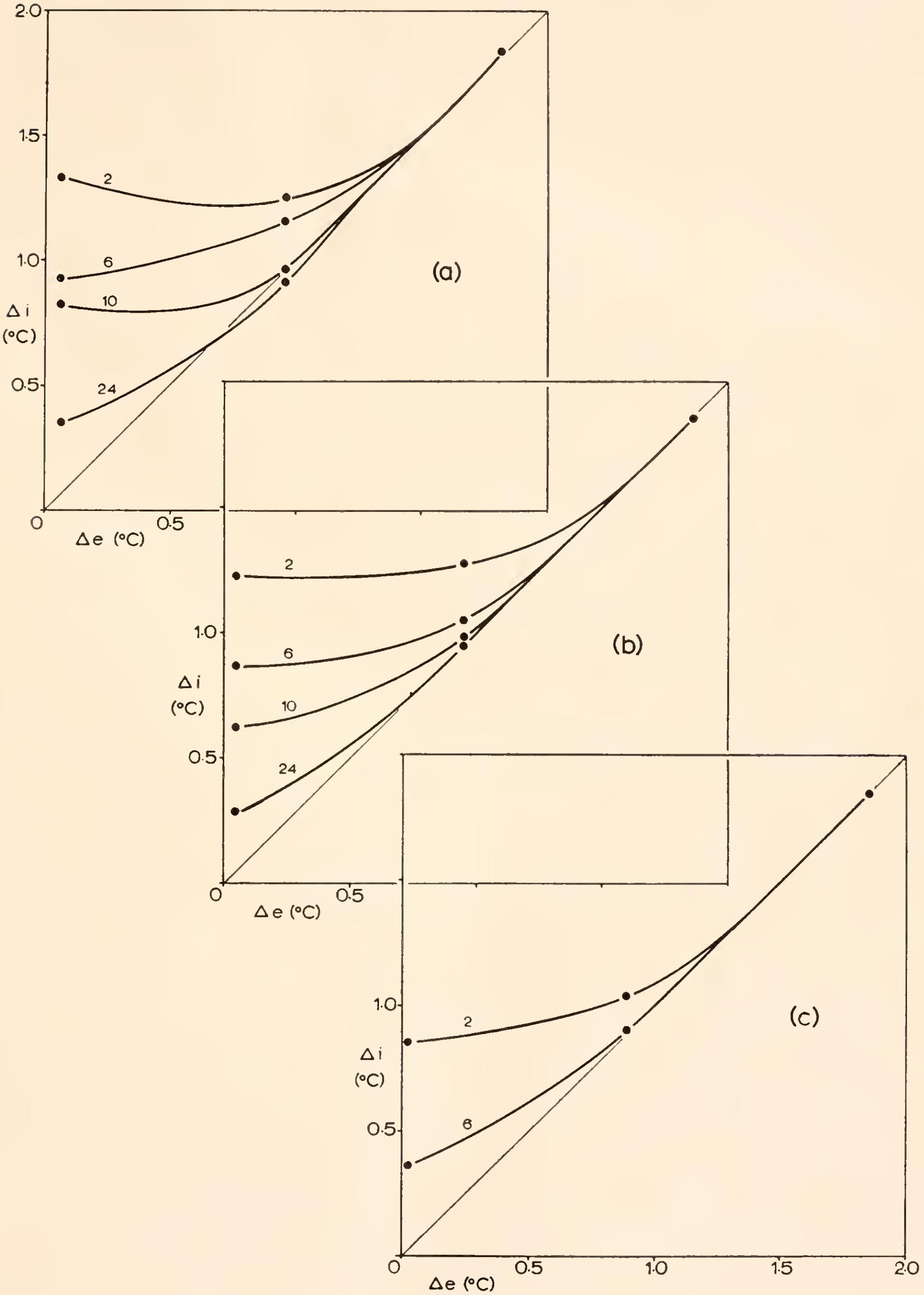

FIGURE 4. Adjustment of pericardial fluid osmotic pressure to that of the external medium at three different temperatures. Time (hours) after transfer to the external medium marked on each curve. (a) 10° C ± 0.1° C; (b) 20° C ± 0.5° C; (c) 30° C ± 0.1° C.

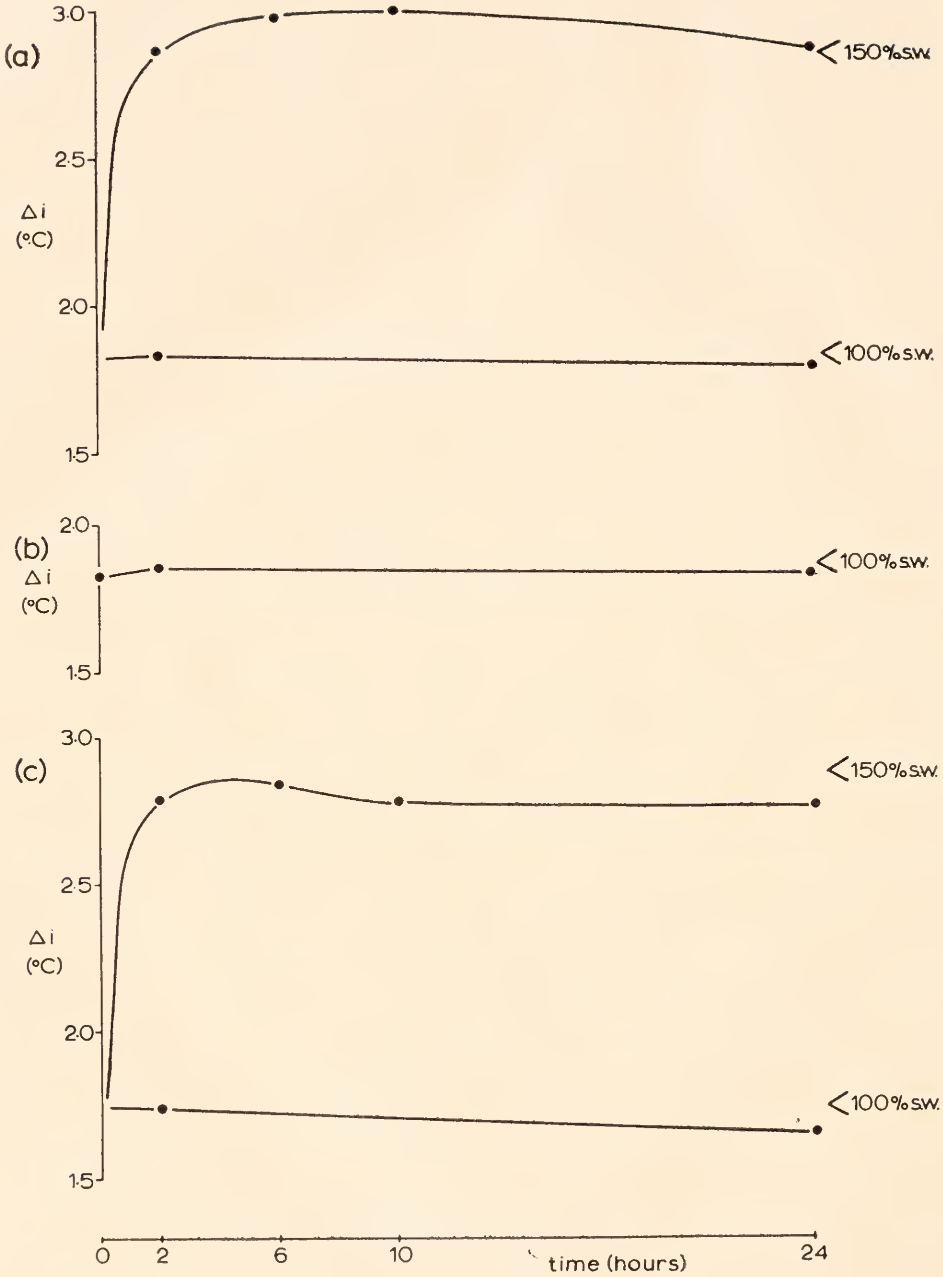

FIGURE 5. Pericardial fluid osmotic pressure in 100% seawater for 24 hours (Controls), and after transfer to hypertonic medium (150% seawater) at two temperatures. (a) 10° C ± 0.1° C; (b) 20° C ± 0.5° C; (c) 30° C ± 0.1° C.

In 150% seawater hardly any movement occurred.

Few faeces were found in tanks of 50% and 0% seawater, while control animals defecated freely. Animals transferred from these conditions to 100% seawater for recovery usually defecated within a few minutes.

DISCUSSION

As all control groups of animals have shown, *Sypharochiton pelliserpentis* is normally in osmotic equilibrium with the surrounding seawater. Differences between the Δi and Δe after 24 hours in normal seawater shown in Figure 5 were found not to be significant ($P = 0.5$).

Unattached animals (Fig. 2) showed a rapid weight increase when transferred to hypotonic media, followed by weight equilibrium and some weight regulation. The effect of firm attachment in reducing the surface area available for diffusion can account both for the lower rate of weight increase, and the greater weight increase eventually reached by attached animals. Firstly the rate of water entry and consequent weight increase is reduced, and secondly salt loss is presumably also reduced so that correspondingly more water enters before weight equilibrium can be attained.

Both attached and unattached animals in dilutions down to 50% seawater seem capable of limited, active weight regulation. In seawater/sucrose mixtures isosmotic with seawater, *S. pelliserpentis* lost weight. This implies salt loss from the animal and probably means that salt loss in dilute media contributes to a passive weight regulation.

There is no evidence that *S. pelliserpentis* is in any way able to regulate its internal osmotic concentration, except by reducing contact with the external medium and thus delaying equilibrium. In 150% and 50% seawaters, osmotic equilibrium was reached rapidly. In 0% seawater, dilution of the internal fluid was very rapid, but mortality occurred before equilibrium was reached. No definite behavioral reaction to a hypertonic medium was recorded. As no mortality resulted from exposure to this medium for up to 24 hours, hypertonicity at this level (150% seawater) was assumed to be of little physiological importance to the animal. Chitons in 150% seawater were easily detached from the substrate, a result presumably of the loss of body fluid. It is probable that loss of body fluid could itself cause loss of locomotory ability.

S. pelliserpentis is able to detect and react to reduced salinities, as can *Chiton tuberculatus* (Arey and Crozier, 1919) and many other molluscs. That clamping down hard to the substrate is more rapid and complete the more dilute the medium, could well account for the situation shown in Figure 1. Here 1 hour after transfer, weight increase was greater in 75% than in 50%, which in turn was greater than in 25% or 0% seawaters.

Weight loss in 150% seawater is smaller than would be expected. Possibly only a limited weight loss can occur, a limit which is quickly reached. This would be possible if blood volume is small (44% in *Cryptochiton stelleri;* Martin *et al.,* 1958), and if water and/or salts are unable to leave the body tissues, which would occur if the tissue cells have a limited permeability, or can otherwise restrict the outflow of water and ions.

In effect, the osmotic response of *S. pelliserpentis* in the absence of any osmoregulatory ability may be considered as a compromise. This chiton is physically well adapted to reduce contact with the environment to a minimum during adverse conditions. Reduction in the rate of osmotic adjustment, coupled with a considerable degree of tolerance of dilution of body fluid is sufficient to ensure survival of the animals for periods of over 10 hours—the maximum exposure time possible. Only very low salinities and high temperature (the least likely combination) could cause mortality in a shorter time. The range of salinity fluctuations recorded in the field (14‰ to 45‰S) suggests that exposure to osmotic extremes is not likely to limit this species in its exploitation of littoral habitats. A limited, active volume regulation is effective in hypotonic seawaters down to about 50% seawater.

This type of osmotic response—with water inflow, some volume regulation, osmotic equilibrium—is typical of many worms, for instance the sipunculid *Dendrostomum zostericolum* (Gross, 1954). Other worms show in addition, osmoregulation; *Onuphis magna* (Ebbs and Staiger, 1965), *Nereis diversicolor* (Jørgensen and Dales, 1955–1957). Some soft-bodied molluscs such as *Aplysia* (Bethe, 1930), show volume regulation, and possibly weak osmoregulation (van Weel, 1957), while others such as *Onchidium* (Dakin and Edmonds, 1931) do not. The habit of mechanically restricting contact with adverse conditions is found commonly in bivalves such as *Mytilus edulis* (Maloeuf, 1938) and *Scobicularia plana* (Freeman and Rigler, 1957), and also the littorinid gastropods *Littorina littorea, L. littoralis* and *L. saxatilis* (Todd, 1964).

The limpet *Acmaea limatula* after weight increases in 50% seawater showed no tendency to return to the initial weight (Segal and Dehnel, 1962). Its increase in body water in 50% seawater was approximately equal to the decrease in 150% seawater. This has been shown here not to be the case in *S. pelliserpentis. Acmaea* did not osmoregulate in salinities from 25% to 150% seawater. The pulmonate limpet *Siphonaria pectinata* is also unable to osmoregulate over a wide salinity range (McAlister and Fisher, 1968). In *Siphonaria* it was shown that undisturbed attachment to the substrate was very important in resisting osmotic stress.

Gilbert (1959) showed that the Δ° C and the composition of the blood of the shore crab *Carcinus maenas* are correlated with the size of the animal. The results summarized in Figure 4 were analyzed for the effect of size on the O.P. of pericardial fluid. No correlation was shown after equilibrium had been reached, nor when the osmotic gradient was steep and the animals still adjusting. These results agree with those for littorinids (Todd, 1964) where it was shown that size had no effect on osmotic balance.

Work with the American West Coast chiton genus *Mopalia* (Barnes, 1965, unpublished student report) has indicated that animals of this genus are osmoconformers in 125% to 50% seawater media.

Sypharochiton pelliserpentis then is a marine animal which on the shore colonizes habitats bringing it into contact with wide salinity fluctuations for short periods of time. In the absence of any extracellular osmoregulatory mechanism, the animal is able to detect salinity changes and react by clamping down to the substrate, isolating its internal fluids from the external medium and reducing the rate of osmotic adjustment. Though capable of a limited active weight regulation, the animal relies for survival primarily on its ability to tolerate, physiologically, considerable swelling and dilution of its internal medium.

I would like to thank Professor J. E. Morton for the use of the facilities of his Department, and his helpful supervision of this work. To Mr. T. A. Turney my thanks are due for advice on the measurement of freezing-point depression, and to Mr. C. Aldridge for the construction of apparatus. I would also like to thank Professor R. F. H. Freeman for his comments and advice.

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SUMMARY

1. The survival, weight changes and pericardial fluid osmotic pressure changes of the chiton *Sypharochiton pelliserpentis*, when subjected to hypo- and hypertonic media (0% to 150% seawater), have been measured. Three experimental temperatures were used, 10, 20 and 30° C, and the size of the animals was taken into account.

2. The chiton was found to be an osmoconformer, its pericardial fluid O.P. adjusting to that of the medium within the range 50% to 150% seawater. It was able to survive these changes for periods of at least 24 hours. In freshwater (0% seawater) dilution of the internal fluid was more rapid, but mortality occurred before equilibrium was reached.

3. Attachment to the substrate is significant in restricting the rate of water entry. The animal detects hypotonic salines and reacts accordingly by clamping down tightly. A limited weight regulation was observed in dilutions down to 50% seawater.

4. Temperature affects the osmotic response, higher temperatures increasing the rate of osmotic adjustment, and the mortality rate.

5. Field measurements suggest that microhabitat osmotic fluctuations are unlikely to limit the distribution of *S. pelliserpentis* on the shore.

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A HISTOCHEMICAL STUDY OF OOGENESIS IN THE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS* [1]

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The sea urchin has an annual reproductive cycle with specific important cellular events occurring at certain times of the year (Caullery, 1925; Fuji, 1960; Holland, 1967; Holland and Giese, 1965; Moore, 1936; Pearse, 1969a, 1969b; Pearse and Giese, 1966; Pearse and Phillips, 1968; Tennent and Ito, 1941). These stages are clearly outlined by Fuji (1960) who split the yearly cycle into five stages: (I) recovering spent, characterized by a few primary oocytes along the wall of the ovary; (II) growing, larger oocytes line the walls; (III) premature, large oocytes along the walls and a few mature ova in the central lumen; (IV) mature, fertile with mature ova; and (V) spent. Not only gametes but also the accessory cells which Holland and Giese (1965) refer to as nutritive phagocytes are found to undergo seasonal changes (Dawydoff, 1948; Holland and Giese, 1965).

Other studies on sea urchin oogenesis deal with the dictyotic stage of Holland and Giese (1965) or what Tennent and Ito (1941) refer to as the diffusion and resting nucleus stage of the primary oocyte when the ovary is in the mature stage of Fuji (1960). These studies are concerned with the synthesis of RNA, protein, and polysaccharides in the nucleolus, and the transfer of these materials to the cytoplasm (Cowden, 1962; Esper, 1965; Ficq, 1962, 1964; Ficq, Aiello and Scarono, 1963; Gross, Malkin and Hubbard, 1965; Piatogorsky, Ozaki, and Tyler, 1967).

Recently, electron microscope studies have been made on sea urchin oogenesis that concentrate mainly on morphological changes of the sex cells and do not deal with seasonal variations (Anderson, 1968; Verhey and Moyer, 1967a).

The present study was undertaken to correlate the morphological and biochemical changes in the egg cells and nutritive phagocytes with relation to the annual reproductive cycle, by histological and histochemical techniques.

MATERIAL AND METHODS

Specimens of *Strongylocentrotus purpuratus* were collected at two week intervals from October 1966 to March 1967 in the tide pools of the mean low tide zone of Yaquina Head, Oregon (latitude 44° 40′ 40″, longitude 124° 4′ 40″). This is the period in which the greatest proliferation of the gonad and the spawning of the eggs takes place. In addition samples of spent ovaries were sampled in April and May of 1966. Although no animals were obtained from June through September, the May and October specimens overlapped sufficiently to present a continuous spectrum.

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The sea urchins were sacrified on the same day as collected or the morning immediately following a night collection. The wet weights of the animals were taken; the urchins were then injected with 2 cc of 0.5 M KCl, and allowed to stand for half an hour to see if they would spawn. Gonads that could not be sexed by macroscopic inspection and all ovaries were removed from the urchins and fixed in Bouin's, Carnoy's or calcium formal fixatives. The fixed material was then imbedded in gelatin for frozen sectioning or in paraffin. The gelatin imbedded tissues were quick frozen and sectioned on a microtome in a Model CTD International Harris Cryostat. Ten micron sections were made of both frozen and paraffin sections.

The hematoxylin and eosin method was used for standard histological examination. Oil red O counterstained with Mayer's hematoxylin was used for neutral lipids; Feulgen reaction, for deoxyribonucleic acid (DNA); methyl green, pyronin (MGP) method was used for ribonucleic acid (RNA) in combination with mild acid hydrolysis of parallel sections, 1 N HCl at 60° C for five minutes, as a control. The periodic acid Schiff (PAS) technique was used for the demonstration of polysaccharides with diastase digestion of parallel sections to indicate the presence of glycogen in the tissue. All histochemical techniques used followed the schedules as outlined in Barka and Anderson (1963). In addition, the presence of RNA was determined by the azure B technique of Flax and Himes (1952) as modified by Szollosi (1965). Mild acid hydrolysis of parallel sections was again used to verify the presence of RNA.

Photomicrographs were made on a Leitz Wetzlar microscope with an Olympia 35 mm camera.

RESULTS

The sea urchin has five separate ovaries; each covered by a flagellated peritoneal epithelium. The ovaries are large rebranched sacs and each saccule ends in a blind acinus. The wall of the ovary under the peritoneum is made up of collagenous connective tissue and smooth muscle. In the central portion of each acinus are two main cell types: the sex cells, which mature into ova, and the accessory cells, also referred to as nutritive phagocytes.

In discussing the yearly reproductive cycle of *Strongylocentrotus purpuratus* the five stages of Fuji (1960) are used. The yearly gross and histological changes are summarized in Table I and temperature and salinity data, in Table II.

Recovering spent stage

This stage occurs, in urchins found along the central Oregon coast, from the late summer months to early fall. There is, however, a great deal of individual variation in the timing of this stage and also in the timing of the other stages. The ovary in the recovering spent stage is small but firm and is slightly larger and more substantial than ovaries that have just been spent. Its color varies from tan to orange depending on the number of dark brown degenerating bodies (discussed below) in the ovary and the amount and size of the immature eggs.

TABLE I

Ovarian cycle

Months most common	Recovering spent I	Growing II	Premature III	Mature IV	Spent V
	Aug.–Oct.	Oct.–Nov.	Nov.–Jan.	Jan.–Apr.	Apr.–Aug.
Oogonia	very few	few	more numerous	numerous	very numerous
Dictyotene oocytes	10–20 μ	20–30 μ	up to 60 μ, all sizes	same	none growing 5–10 μ
Ova	none	none	few	many	degenerating
Nutritive phagocytes	lightly globulated	heavily globulated	same	emptying	empty and refilling
External appearance	dark brown firm, small	brown to orange, firm medium	brown to orange "fluffy" full size	orange, ripe full size	dark brown flaccid, small

In histological section the nutritive phagocytes completely fill the entire ovary, obscuring the lumina except for the small oocytes that abut against the wall of the ovary. These nutritive phagocytes contain eosinophilic droplets (Fig. 1), but the droplets are not as densely packed nor as numerous as in later stages. The globules stain very intensely with the periodic acid Schiff method, indicating large amounts of polysaccharides (Figs. 3 and 4). Glycogen extraction only reduces the intensity of the stain evenly over the entire ovary, indicating that while glycogen may be present in large amounts, other polysaccharides may make up the greater percentage of these globules or the glycogen is some how protected from the action of the enzyme. These cells also contain many lipid inclusions (Fig. 2), and their nuclei are Feulgen positive.

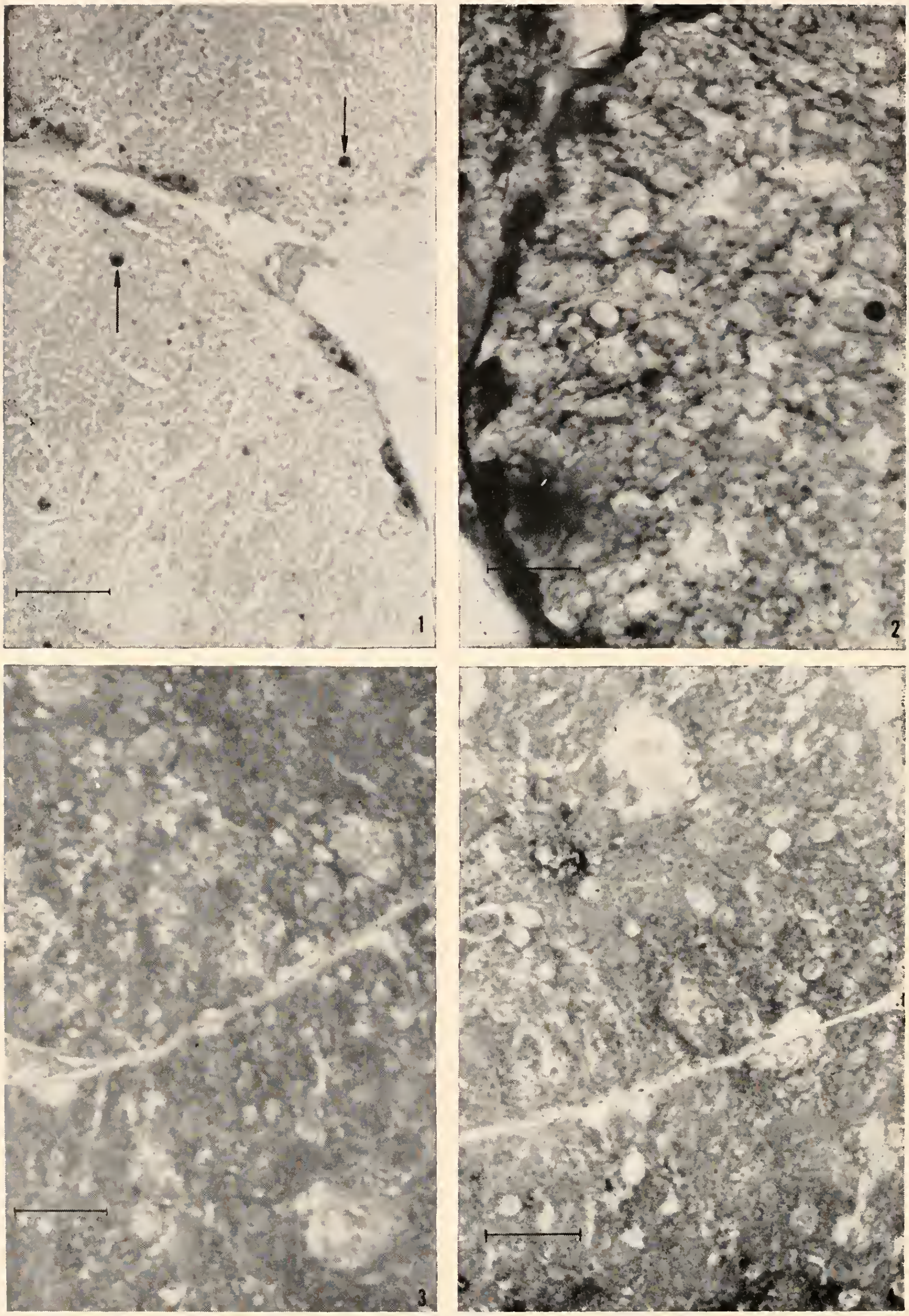
Among the nutritive phagocytes many sex cells can be seen with an irregular or indistinct outline. Also, dark brown granules (Figs. 1 and 5) and numerous basophilic inclusions (Fig. 6) are present, although the granules and inclusions are somewhat more common in later stages. The brown granules are Feulgen positive, indicating they contain DNA and are probably breakdown products of nutritive

TABLE II

*Temperature and salinity ranges**

	Aug.–Oct.	Oct.–Nov.	Nov.–Jan.	Jan.–Apr.	Apr.–Aug.
Surf temperature °C Newport 1966	10–15	10–14	9–12	8.5–11	8–16
Surf temperature °C Depoe Bay 1967	13–18	10–13	10–12	9–11	11–18
Salinity ‰ Newport 1966	32–34	29–33	22–32	27–33	28–33
Salinity ‰ Depoe Bay 1967	28–34	32–34	32–34	29–32	28–34

* Temperature and salinity data from Wyatt and Gilbert, 1967 and Gilbert and Wyatt, 1968.



Figures 1–4.

phagocytes, developing eggs or perhaps both (Fig. 7). There are often other basophilic granules in or between the nutritive phagocytes that contain concentrations of RNA and occasionally others which contain both DNA and RNA.

Oogonia are very difficult to find in the recovering spent ovary; they occur singly or in very small groups scattered along the wall of the ovary. These oogonia, whose diameter is an average of five microns, have a scanty cytoplasm that does not stain specifically. Generally, two small dense nuceoli distinguish them from primary oocytes of the same size. The nuclei of the oogonia are slightly Feulgen positive.

Many small primary oocytes just beginning their growth or dictyotic phase, resting or diffusion nucleus stage of Tennent and Ito (1941), can be seen along the wall of the ovary (Fig. 1). There is generally an interval of about 15 microns between each one and they are slightly elongated in their axis parallel to the ovarian wall with their narrowest width ranging from 10–20 microns. The cytoplasm of these small oocytes shows a great concentration of azure B positive material which does not stain with pyronin (Fig. 6). Although some polysaccharide is present in the cytoplasm of the oocytes at this stage, it is very scant compared with the dense concentration in the adjacent phagocytes. No lipid appears in the oocytes at this time and no Feulgen stain can be seen in the germinal vesicle.

The most prominent feature of the young oocytes is the nucleolus, which is three to five microns in size. At this stage it often appears to be more eosinophilic than the cytoplasm, for it contains not only material stainable with both pyronin (Fig. 8), and azure B (Figs. 6 and 14), but also diastase extractable and non-diastase extractable polysaccharides. Despite this the nucleolus appears very dense and homogeneous during this stage.

Growing stage

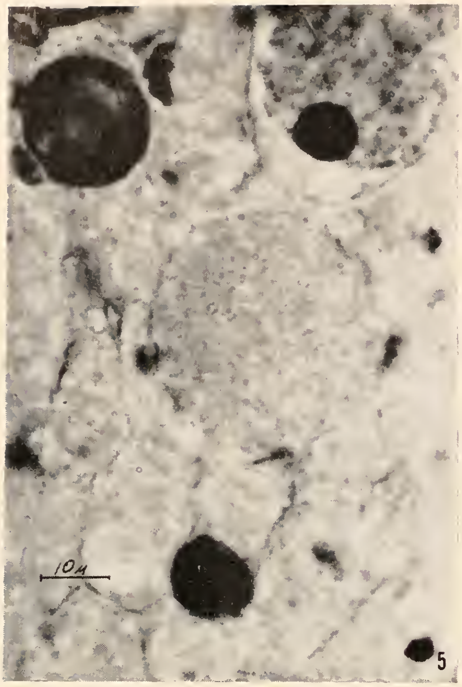
In the late fall the ovaries enter the growing stage during which time they greatly increase in size. (See gonad index for *S. purpuratus* at Coos Bay, Boolootian, 1966.) They have a mealy texture and vary in color from a tan to the golden orange color of mature eggs. At the cellular level, the nutritive phagocytes are densely filled with polysaccharide and lipid globules (Figs. 9, 10, 11, and 12), and large vacuoles are present in many of these cells (Fig. 12). The phagocytes have attained their maximum size of 15–20 microns in diameter, and are irregular in shape due to crowding. Their cytoplasm is pyroninophilic at this stage (Fig. 13) but only a barely perceptible bluing occurs with azure B. Many oocytes seem to have indistinct borders where they abut with phagocytes, although this is difficult to ascertain exactly with the light microscope (Figs. 14 and 15).

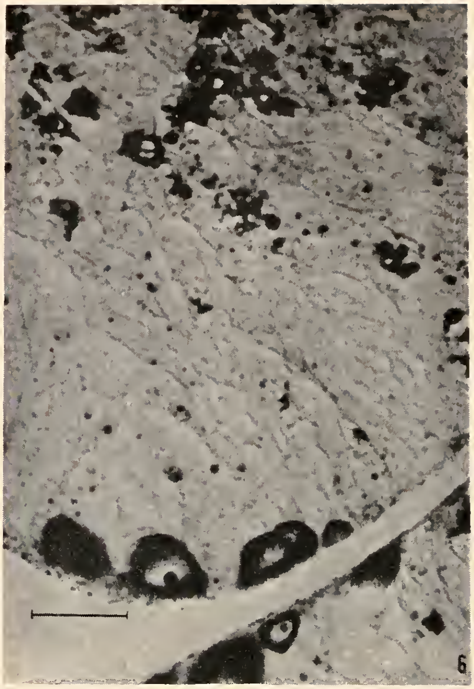
FIGURE 1. Acinus of late recovering spent ovary. Note small primary oocytes along walls. Accessory cells (nutritive phagocytes) are filled with eosinophilic globules and have large dark granular inclusions (arrows). Hematoxylin and eosin (H&E). Micron markers on micrographs represent 50 microns unless otherwise indicated.

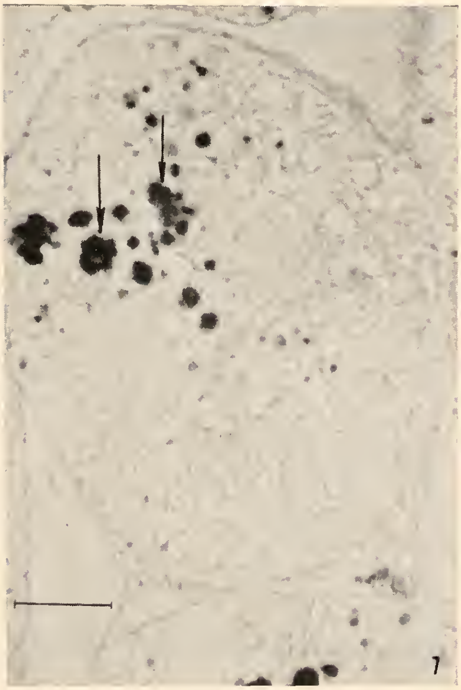
FIGURE 2. Late recovering spent ovary. Nutritive phagocytes take up lipid stain. Small oocytes along ovarian wall appear very dark and stain only with hematoxylin. Gelatin imbedded; oil red O and Mayer's hematoxylin (H&O).

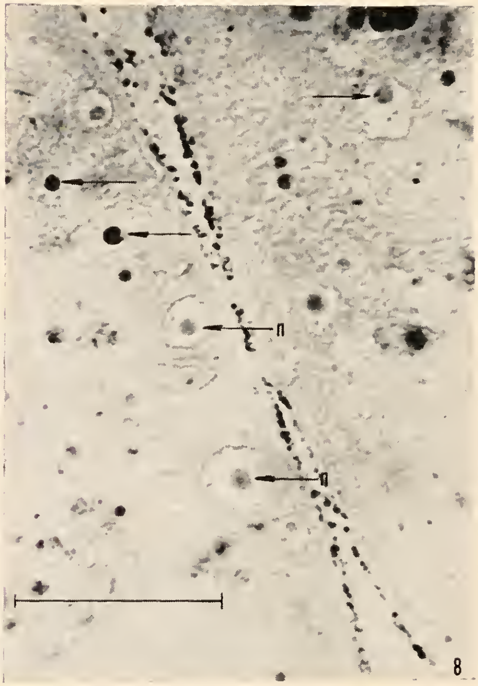
FIGURE 3. Late recovering spent ovary. Accessory cells full of polysaccharides. Periodic Acid Schiff (PAS).

FIGURE 4. Parallel section to one in Figure 3 with glycogen extracted with diastase. PAS.









FIGURES 5–8.

The oocytes have attained a size of 20–30 microns in diameter; most are slightly elongated in the axis perpendicular to the acinar wall, while some are spherical. The cytoplasm of these oocytes is similar to that described in the previous stage, but the azure B stain is not as intense.

On the other hand, a slight change has occurred in the nucleolus; it is more intensely pyroninophilic than is the previous stage and often possesses one or two small vacuoles (Fig. 14). It has not kept pace with the growth of the rest of the cell and appears to be the same size as it was in the previous stage. Scattered particles appear in the germinal vesicle that stain with hematoxylin in the same way that the nucleolus does, but these particles do not stain with azure B, pyronin, or PAS.

Premature stage

In this stage, which occurs in the early winter, the ovaries are golden orange and take on the outward appearance of mature ovaries. Although there are a few ripe eggs present in the ovary, they will not be shed even with the stimulus of KCl injections. The nutritive phagocytes remain much as described in the previous stage, but those in the more central portions of the ovary have lost their pyroninophilia. Nests of oogonia and primary oocytes in the pre-dictyotic phases are now quite frequent (Fig. 16).

The uniformity in size of the growing oocytes is lost, for all sizes can be found from the smallest to the largest which are about 50–60 microns in diameter, the size of the mature ovum. The smaller oocytes are usually found in the peripheral acini. The cytoplasm of these small oocytes stains much more intensely with azure B than that of larger oocytes (Fig. 17), while the larger oocytes contain much more polysaccharide (Fig. 18), and lipid appears in their cytoplasm for the first time (Fig. 19). Although these two constituents are also found in the globules of nutritive phagocytes, they occur in the cytoplasm of the oocyte as much finer grains. The nucleolar vacuoles become larger and more numerous and make up a large portion of the nucleolar area in the biggest oocytes. Usually these vacuolated nucleoli have lost their pyroninophilia, but still show visible staining with the azure B and the PAS techniques.

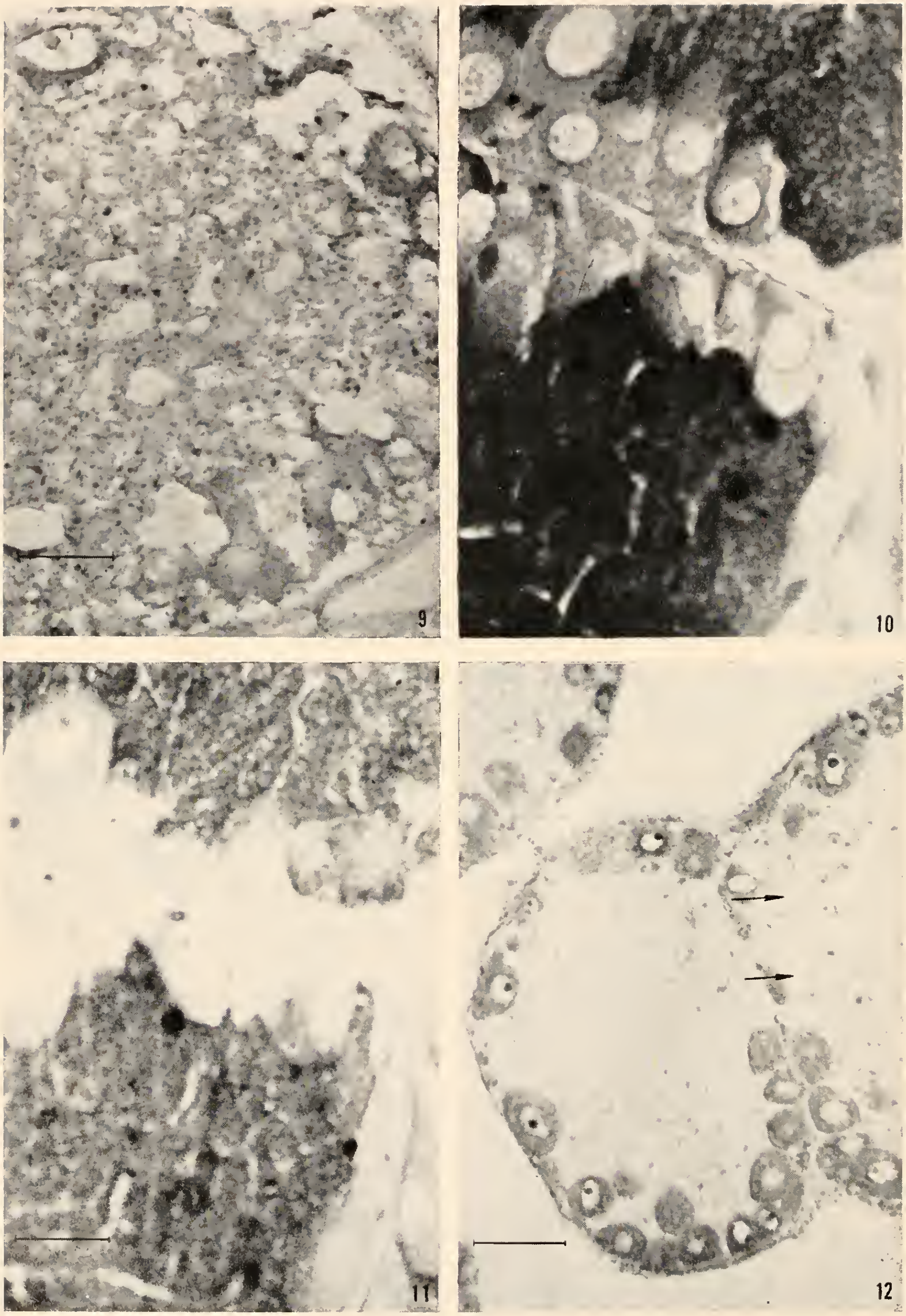
At the time of the maturation divisions the germinal vesicle becomes ill-defined as its membrane breaks down and the nucleolus disappears. The oocyte either moves or is pushed toward the center of the acinus where meiosis takes place. This movement displaces the nutritive phagocytes that had formerly been in the center, but no patent lumen is formed. The spindle of the maturation divisions forms near the plasma membrane on one side of the egg, thus producing small polar bodies.

FIGURE 5. Late recovering spent ovary. Inclusions of nutritive phagocytes. Note large dark granular inclusions and inclusion that looks like the germinal vesicle of an oocyte with its nucleolus in upper right corner. H&E.

FIGURE 6. Late recovering spent ovary. Cytoplasm of small oocytes stain dark blue indicating the presence of RNA. Basophilic inclusions of nutritive phagocytes (top of picture) stain purple. Azure B.

FIGURE 7. Mature ovary. Large dark granular inclusions (arrows) are Feulgen positive. Feulgen.

FIGURE 8. Growing ovary. Nucleoli (n) of germinal vesicle are pyroninophilic. Large dark granular inclusions stain with methyl green (arrows). Methyl green and pyronin (MGP).



Figures 9–12.

As the season progresses the ovary becomes studded with the small pyknotic nuclei of the polar bodies, which can be identified most readily with the Feulgen stain. Few eggs can be found undergoing maturation divisions at any one time and it is often necessary to hunt carefully in serial sections to find any at all. The nucleus of the mature ovum is very small, about five microns in diameter, as compared to the germinal vesicle of the oocytes which is 20–25 microns in diameter. The chromosomes, which are now concentrated enough to be visibly stained by the Feulgen method, can be seen as small droplet-like areas adhering to the nuclear membrane.

Mature stage

The ovaries usually become mature about midwinter and the breeding season commonly lasts well into the spring. The ovaries are colored a golden orange by the mass of mature ova which they contain. They will shed after almost any mild shock and with KCl they will shed almost immediately.

There is often a large pyroninophilic area in the ova that are about to be shed and those that have already been shed. While still within the ovary this area is irregularly shaped, as are the ova themselves due to crowding, but once the ova are released both they and the pyranophilic area within them assume their normal spherical shape (Fig. 20).

The mature ova within the ovary have displaced the nutritive phagocytes to the outside of the acinus where they form a single layer containing oocytes that have not yet matured (Fig. 21). The more peripheral acini retain an aspect similar to that found in the premature ovaries, except that there tends to be a great variation in the sizes of the oocytes (Fig. 22). Usually the smallest oocytes, ten microns in diameter, occur in clusters in association with one or more nests of oogonia and pre-dictyotic oocytes, all of which become more frequent as the season progresses.

Due to the presence of continually growing oocytes the urchin is able to shed several times during the breeding season. A definite, progressive change can be noted in the phagocytes over this time. The change starts with the cells in the more central portion of the ovary and proceeds toward the more peripheral acini over the course of the season. The lipid and polysaccharide droplets within these cells become fewer in number (Fig. 23) and eventually are entirely depleted in the spent stage (Fig. 24). At this point some of the phagocytes disappear, but many remain, identifiable only by their plasma membrane, small nucleus, and a few dark granular inclusions.

Toward the end of the breeding season the size of the oocytes becomes progressively smaller as less cytoplasm is produced. They are elongated as opposed

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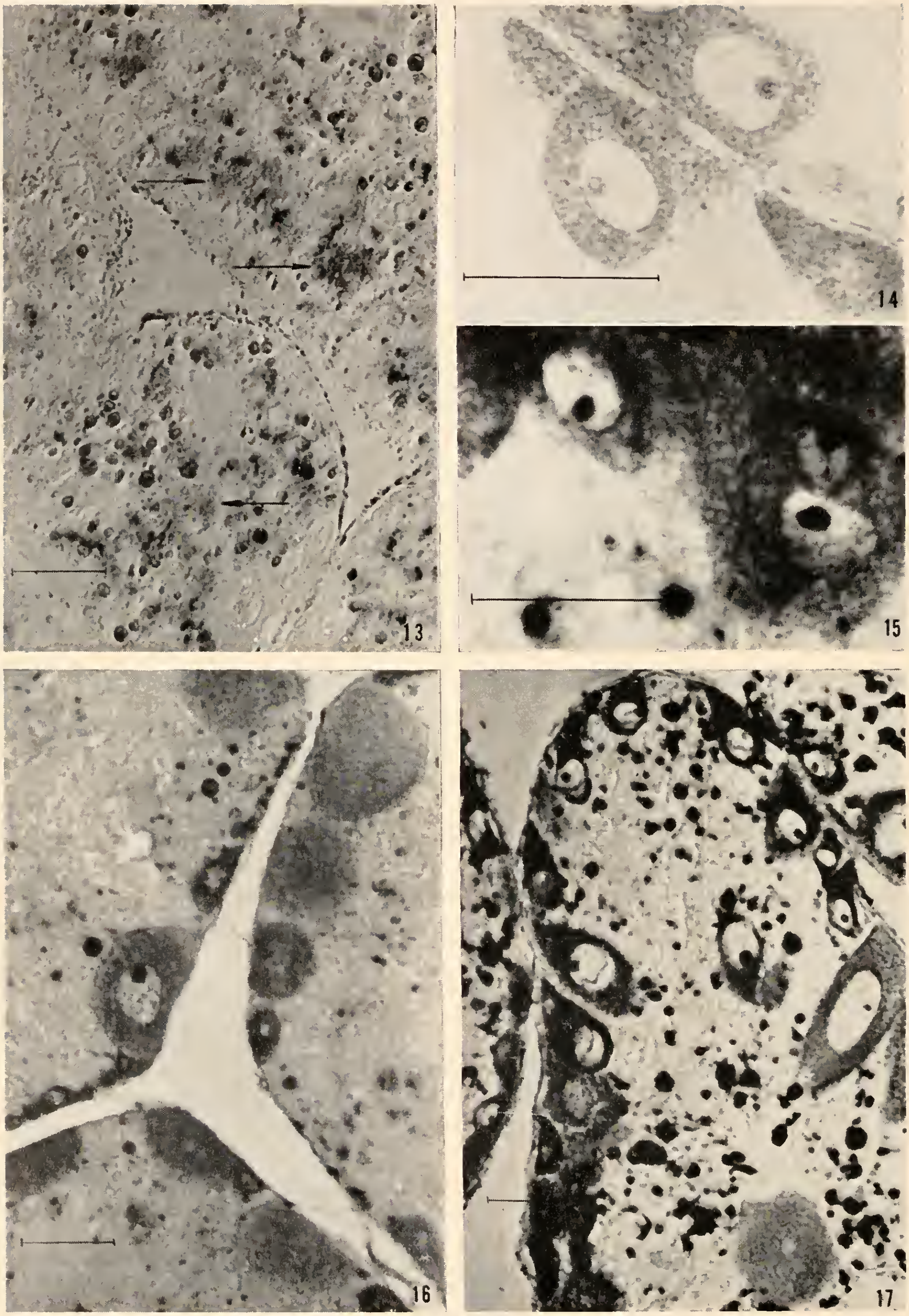
FIGURE 9. Growing ovary. Nutritive phagocytes stain with oil red O and oocytes stain with hematoxylin. H&O.

FIGURE 10. Growing ovary. Oocytes do not stain as darkly as the surrounding accessory cells. Note nucleolus of germinal vesicle is PAS positive. PAS.

FIGURE 11. Parallel section to one in Figure 10 with glycogen extracted with diastase. PAS.

FIGURE 12. Growing ovary. Individual eosinophilic globules can no longer be distinguished as they can in Figure 1. Note vacuoles in nutritive phagocytes (arrows). H&E.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16
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Figures 13–17.

to the spherical or elliptical shape common earlier in the season (Fig. 24). Usually these oocytes are not shed even if they do mature and the ovary is now termed spent.

Spent stage

At this time the external aspect of the ovary looks very much reduced, flabby, and has a dark brown color due to the presence of brown degenerating bodies. The nutritive phagocytes are empty, giving the inside of the ovary an open mesh appearance. Quite a few deteriorating, unshed ova can be seen in the now open lumina (Fig. 25).

One very interesting and important event occurs at this time: the oogonia, pre-dictyotene oocytes, and very small dictyotene oocytes that have been slowly increasing in numbers over the course of the year are now quite numerous (Fig. 25). Together these various types of germ cells form a layer of two or three cells thick just under the wall of the acini. As fall approaches the number oogonia and oocytes decreases, but some of the small oocytes remain and begin growing along the wall of the ovary (Fig. 1). The nutritive phagocytes begin refilling with globules as the ovary once again enters the recovering spent stage.

DISCUSSION

A study of the annual reproductive cycle of the sea urchin shows that there is no period of the year when the ovary can be considered dormant. The cycle can roughly be divided into two parts: the first when the phagocytes are filling with globules and the majority of sex cells are in the dictyotene phase, and the second when the phagocytes are being depleted of their globules and sex cells in stages earlier than the dictyotene phase are numerous. Since the large growing oocytes are most plentiful at that time of year when the phagocytes are full of globules it seems that the globules are necessary for oocyte growth, especially since polysaccharide and lipid appear in the phagocytes before they are seen in the cytoplasm of the oocytes.

Although indistinct borders do occur between phagocytes and growing oocytes as seen with the light microscope (Figs. 14 and 15), it is unclear whether this represents an engulfment of the oocyte by the phagocyte or active transport in the reverse direction. While Miller and Smith (1931) report incorporation of degenerating accessory cells by the oocytes in *Echinometra lucunter,* the present author concurs with Holland and Giese (1965) in that no phagocytosis of the nutrive cells by the oocytes was observed in *S. purpuratus.* Takishima and Takishima (1965) report that glycogen particles in the ovaries of *Hemicentrotus pul-*

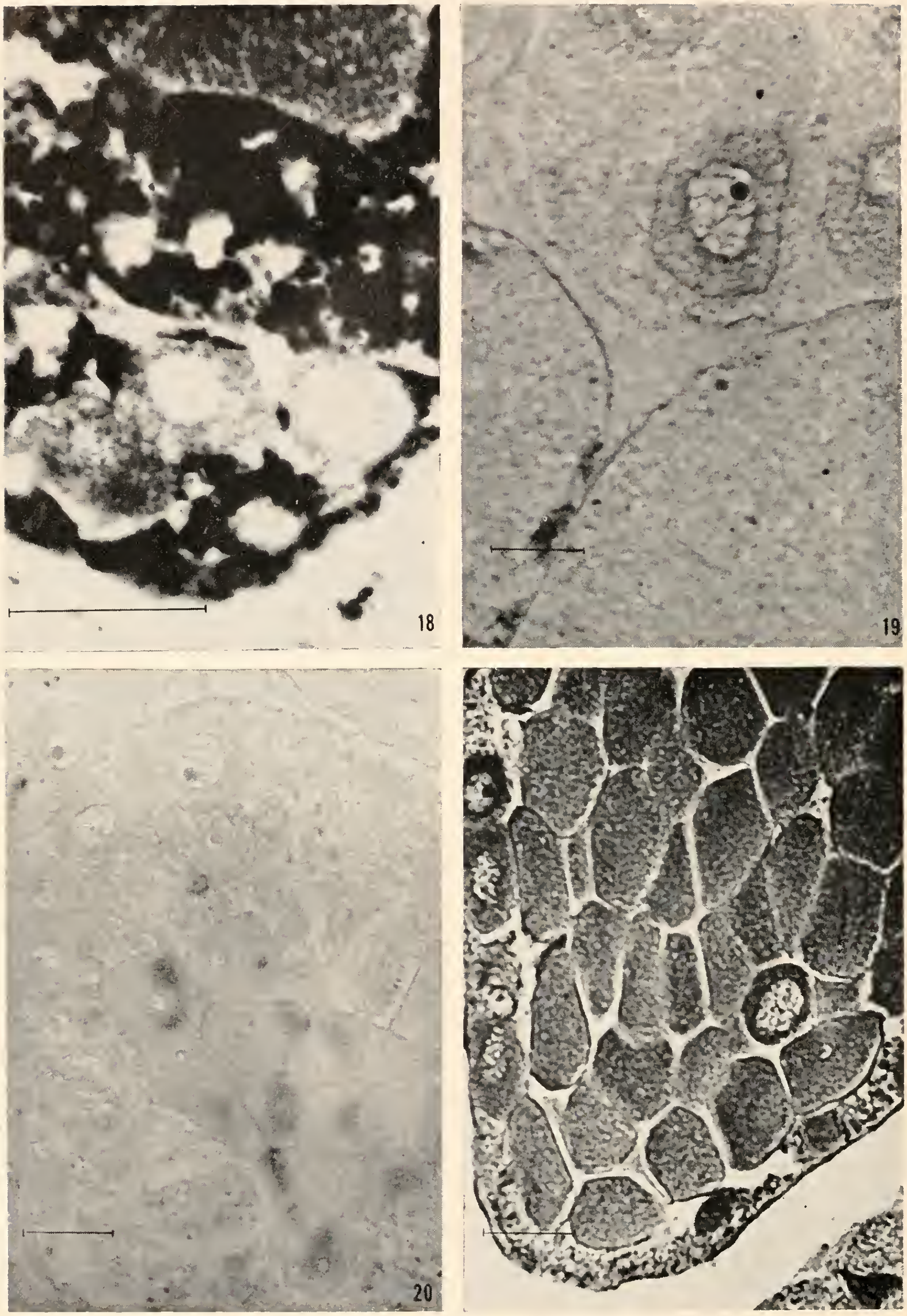
FIGURE 13. Growing ovary. Nutritive phagocytes have pyroninophilic areas (arrows). Dark granular inclusions are stained with methyl green. MGP.

FIGURE 14. Growing ovary. Primary oocytes in dictyotic stage. Nucleoli contain vacuoles. Note border of the upper large oocyte is indistinct. Azure B.

FIGURE 15. Growing ovary. Primary oocytes with indistinct boarders. H&E.

FIGURE 16. Premature ovary. Note variety in size of egg cells lining the wall of the acinus. H&E.

FIGURE 17. Premature ovary. Smaller oocytes stain more intensely with stain for RNA than larger ones and ova. Basophilic inclusions in nutritive phagocytes similar to those in Figure 6. Azure B.



FIGURES 18–21.

cherrimus are released by the accessory cells into an intercellular space and that these particles are in turn taken up by the oocyte by pinocytosis. However, Verhey and Moyer (1967a) report that no pinocytotic vesicles occur in the plasma membrane of the oocytes of *Arbacia punctulata, Lytechinus variegatus,* and *L. pictus* and concluded that RNA and proteins elaborated in the nutritive phagocytes are not taken up by the oocytes; however these authors did not study the oocytes over the course of the year, and it may be that pinocytosis oocurs at a stage during the annual reproductive cycle other than the one they investigated. The same authors (Verhey and Moyer, 1967b) also found none of the polysaccharide in the accessory cells of the sea urchins they examined was glycogen. On the other hand *S. purpuratus* has large amounts of glycogen in its accessory cells as well as polysaccharide that is not extracted with diastase (Figs. 3, 4, 10, 11 and 18). Even this non-extracted polysaccharide may be glycogen that is somehow protected from the action of the diastase.

While it is not clear if the sex cells obtain nutriment directly from the phagocytes, there is evidence, on the other hand, that the phagocytes engulf sex cells. Fuji (1960) reports absorption of unshed degenerating ova, and Pearse (1969b) reports breakdown and subsequent phagocytosis of smaller oocytes when larger ones nearing maturity are numerous. Many phagocytes in the recovering spent phase contain RNA and DNA positive inclusions that under close inspection with the light microscope appear to be degenerate nuclei of early dictyotene oocytes (Fig. 5). Certainly this mechanism would account for the drastic reduction from the large number of immature sex cells seen along the walls of the spent ovary to the amount seen in the early growing stage. This raises the question of whether the phagocytes exercise some control on the number of oocytes that can develop. Alternatively, Holland (1967) suggests that growing oocytes might have an inhibitory effect on smaller primary oocytes.

The cyclical appearance and disappearance of the globules within the phagocytes poses another important question. If the oocytes do indeed derive nutriment from the phagocytes, it is easy enough to explain their deglobulation. However, it is unlikely that the phagocytes are completely refilled by incorporating degenerating ova or developing oocytes for there are not enough of these to account for the abundance of polysaccharide and lipid that appears within them during the globulated stage. It is more reasonable to suppose that this variation is due to greater availability of food in the animals' habitat during the time when the phagocytes are filling and its relative scarcity when they are becoming depleted. Fluctuation in the food supply may likewise explain the great variability in gonad size and fertility from year to year (Boolootian, 1966). This however, is probably not the full answer, for Japanese urchins show a decline in food consumption during the premature and mature stages which does not correlate with a decline in

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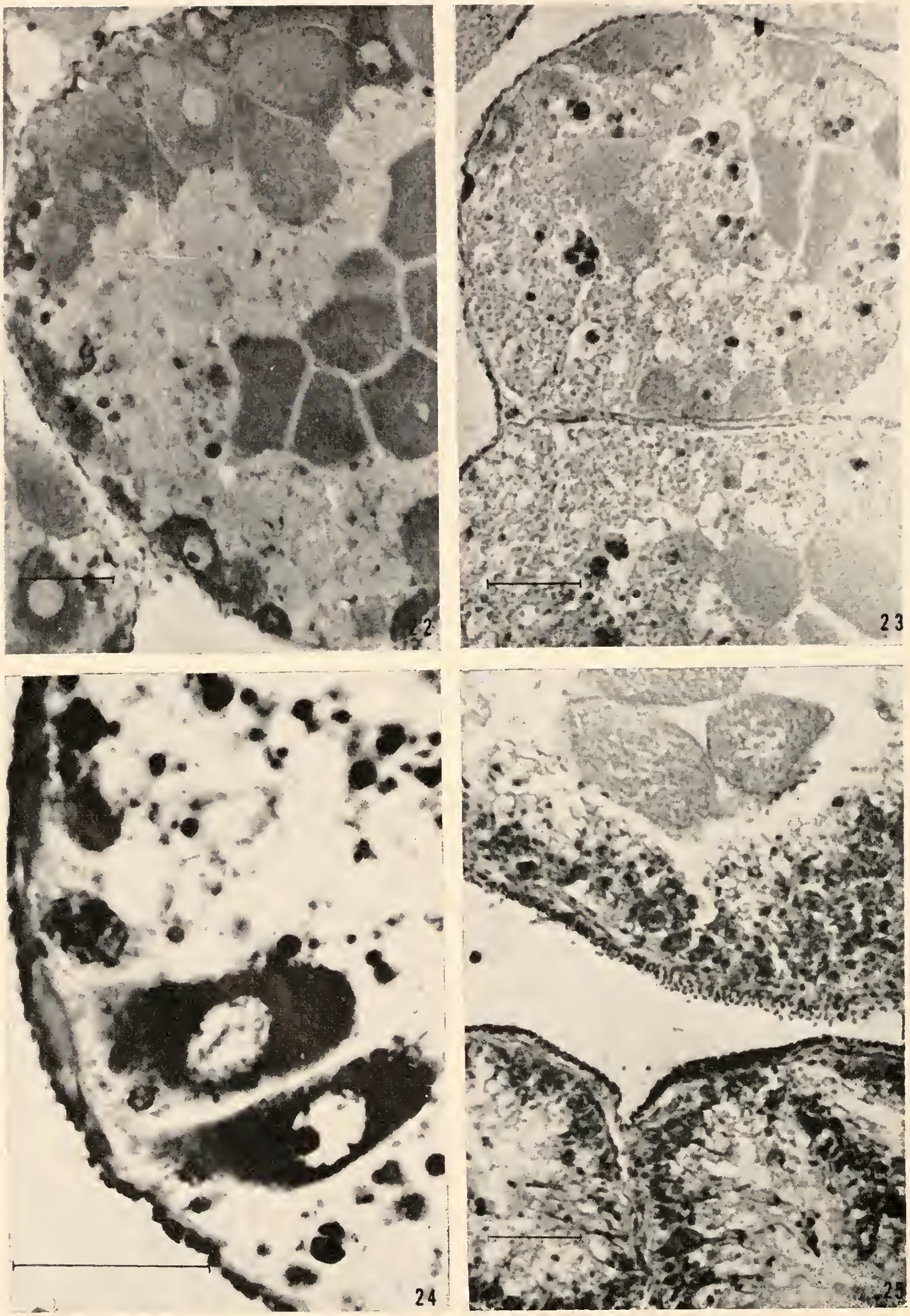
FIGURE 18. Mature ovary, peripheral acinus. Oocytes do not stain as intensely as ova. PAS.

FIGURE 19. Premature ovary. Oocytes appear purple because they stain with both lipid stain and hematoxylin. Nucleolus of germinal vesicle stains only with hematoxylin. H&O.

FIGURE 20. Mature ovary. Pyroninophilic area occurs in ova. MGP.

FIGURE 21. Mature ovary, central acinus. The numerous ova "push" nutritive phagocytes and growing oocytes into a single layer against the wall of the acinus. Ova are irregular in shape due to crowding. H&E.



Figures 22–25.

food supply (Fuji, 1967). Holland (1967), studying *Stylocidaris affinis*, thought photoperiod might be used as a reference point to synchronize an endogenous reproductive rhythm.

It has also been suggested (Giese, 1959) that temperature and salinity may have some influence on the timing of the reproductive cycle. From the data available (see Tables I and II) there seems to be a correlation between the filling of the phagocytes and temperature, but none with salinity. The water temperatures and salinities recorded are those of the open surf and would be correct for subtidal urchins; however, the intertidal ones caught in the tide pools would probably be exposed to much higher temperatures and increased salinity due to evaporation.

The seasonal variation in the amount of oogonia and pre-dictyotene oocytes raises the question of where the additional oogonia come from. Tennent and Ito (1941) suggested that they were derived from the peritoneal epithelium, but they were unable to present any proof. Although the incorporation of DNA precursors was followed over the course of one year (Holland and Giese, 1965), this study did not give any support to Tennent and Ito's theory and in fact indicates that oogonia are derived from previously existing ones by mitosis. The cells of the visceral epithelium are highly differentiated flagellated cells of the type described by Lyons, Bishop, and Bacon (in preparation) and occur commonly in other parts of the visceral epithelium as well. It seems unlikely that these cells would redifferentiate into germ cells.

No positive Feulgen reaction is seen in the germinal vesicles of the dictyotene oocytes because the DNA is presumably too dilute to be detected by this method. In the mature egg a Feulgen reaction can again be seen for the chromosomes have recondensed as small droplet-like areas adhering to the nuclear membrane. This configuration is apparently characteristic of sea urchins for it has also been reported by Burgos (1955) in *Arbacia punctulata* and by Agrell (1958, 1959) in *Paracentrotus lividus, Arbacia lixula,* and *Spantangas parcus.*

The discrepancies between the results obtained with the pyronin and azure B methods for staining RNA can be explained if it is possible that they are staining different species of RNA. It must be remembered that the basis of the behavior of these two staining techniques has not been definitely worked out, and therefore the following discussion is conjectural. However, from what is presently known about these two stains it is possible to assume they act somewhat differently. Immers (1961) stained unfertilized sea urchin eggs with pyronin and stated that if the phosphate groups of RNA are combined with protein as they are when the RNA is actively synthetic, the RNA will not take up the stain. On the other hand Flax and Himes (1952) state that azure B competes with protein for the phosphate groups of RNA and therefore is apparently able to stain RNA that would normally be combined with protein by replacing the protein. If this is true, then the azure B stains RNA that is more highly saturated with protein and there-

FIGURE 22. Mature ovary, peripheral acinus. Note variety in size of sex cells. H&E.

FIGURE 23. Mature ovary, toward end of breeding season. Individual eosinophilic globules can once again be seen in nutritive phagocytes. H&E.

FIGURE 24. Spent ovary. Large oocytes are elongated. No eosinophilic globules occur in nutritive phagocytes, only dark granular inclusions. Many very small primary oocytes and oogonia occur along acinus wall. H&E.

FIGURE 25. Spent ovary. Degenerating ova in open lumen. Great proliferation of very small primary oocytes and oogonia along wall of acinus. H&E.

fore presumably synthetically active while pyronin stains the relatively inactive RNA that is not highly saturated with protein. On this basis the growing oocytes contain actively synthetic RNA, while the RNA found in the nutritive phagocytes is mainly inactive. Alternatively, this staining phenomenon may have something to do with the fact that some of the RNA is blocked by a protein inhibitor while the rest is not. The appearance of pyroninophilia in mature ova (Fig. 20) may correspond to the inactive form of messenger RNA said to be present in unfertilized sea urchin eggs (see reviews by Grant, 1965; Gross, 1967; Spirin, 1966). The position and time of appearance of the pyroninophilia may correspond to the appearance of the heavy bodies (Afzelius, 1957; Harris, 1967) for it rarely occurs in the oocytes before they have undergone the maturation divisions and likewise heavy bodies do not appear until this time (Verhey and Moyer, 1967a. The captions of Figs. 19 and 20 of their report refer to ova as mature oocytes which makes interpretation of their electron micrographs rather confusing).

Chaet (1966) and Scheutz and Biggers (1967) have shown that there is a hormone that can be extracted from the radial nerve of the starfish, *Asterias forbesi,* that induces germinal vesicle breakdown and subsequent maturation division. It is possible that a similar mechanism may be at work in sea urchins and it may also cause the oocytes to migrate from the ovarian wall to the central lumen. Another possibility is that the larger oocytes may be capable of limited ameoboid movement that is largely suppressed by the crowding of the nutritive phagocytes. When the phagocytes are smaller and deglobulated at the end of the breeding season, they no longer suppress this ameoboid movement and the oocytes take on a very elongated shape (Fig. 24).

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SUMMARY

1. Oogenesis in the sea urchin *Strongylocentrotus purpuratus* was studied by histological methods and by histochemical techniques for polysaccharides, lipids, and nucleic acids. Urchins were collected at Yaquina Head, Oregon at regular intervals between April 1966 and March 1967. An attempt was made to correlate seasonal variations in coastal water temperature with the gonadal cycle.

2. Oogonia can be found throughout the year in small groups scattered along the walls of the ovary, but are most numerous in the late spring and early summer when the ovary is spent. The oocytes start growing in the late summer and early fall when the accessory cells start filling with lipid and polysaccharide globules. At this time the accessory cells are found to have inclusions that appear to be degenerate sex cells. The oocytes continue to grow through the late fall and early winter and their cytoplasm fills with lipid and polysaccharide. As the ova mature they move from the wall to the central portion of the acinus where they displace the accessory cells that had formerly been there.

3. The ova that have been shed or are about to be shed contain pyroninophilic RNA which is not found in the cytoplasm of the oocytes. However, both ova and

oocytes have RNA that is stainable with azure B. The pyroninophilic RNA is also found in accessory cells.

4. Since all the oocytes do not mature at the same time, a sea urchin is able to shed many times during the breeding season which lasts from late December to early April. During this period the accessory cells progressively lose their globules. When the accessory cells are finally depleted of their lipid and polysaccharide, the oocytes no longer grow and the ovaries are spent.

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HISTOLOGY OF THE PYLORIC CAECA AND ITS CHANGES DURING BROODING AND STARVATION IN A STARFISH, *LEPTASTERIAS HEXACTIS*

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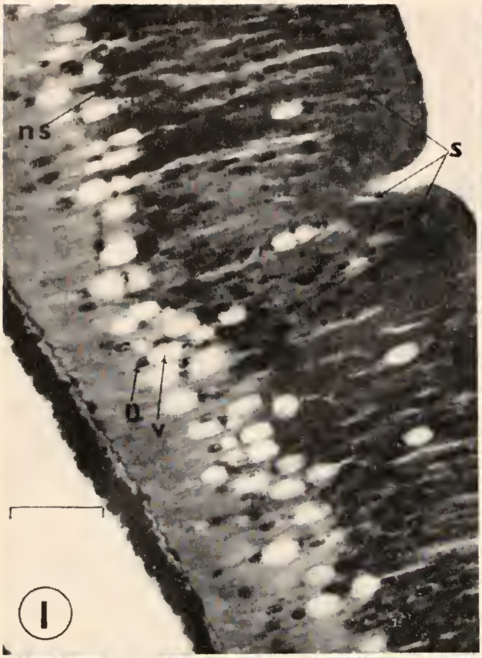
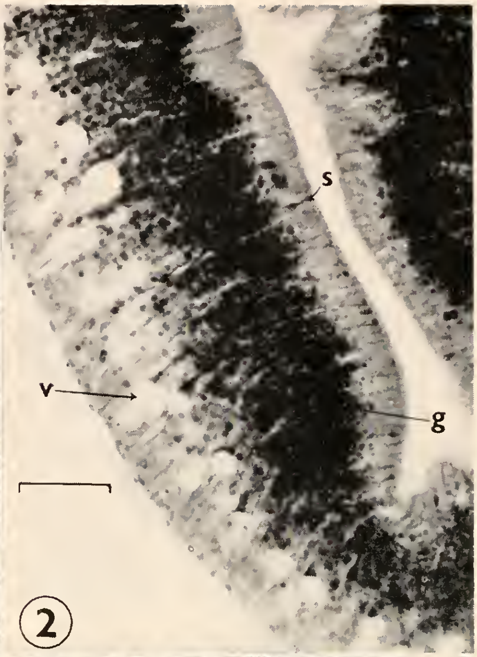
Leptasterias hexactis, the six-rayed starfish, stops feeding completely during more than two months of brooding. Yet, throughout this period, the animal not only spends energy on self-maintenance and care of its young, but it also supports the continuous growth of its oocytes (Chia, 1966, 1968). In other words, the various energy expenditures during brooding have to be derived from nutritional reserves. The cessation of feeding, however, must be considered as behavioral or physiological phenomena because, despite the depletion of nutritional reserves, the animals do not give up brooding and feed, nor are the embryos which are brooded just outside the mouth eaten. It should be recalled that in a closely related species, *Leptasterias groenlandica,* the animals actually brood their young in the cardiac stomach and these are not digested (Lieberkind, 1920; Fisher, 1930).

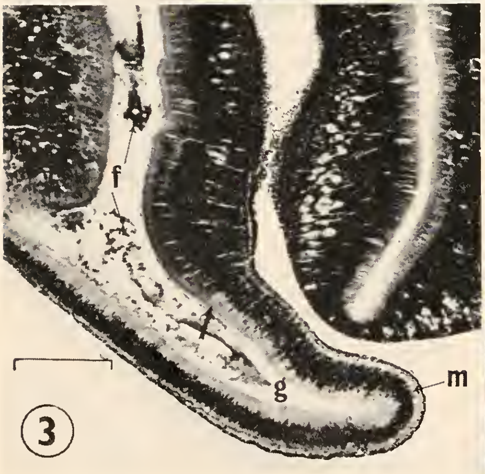
In considering these questions, a comparative histological study of the pyloric caecum in pre-brooding (feeding), brooding and starved animals is useful, since this is the chief organ for absorption, secretion and storage (see review by Anderson, 1966).

MATERIALS AND METHODS

Both brooding and pre-brooding animals were collected from Friday Harbor, Washington, during the breeding season (winter months) of 1962. The pyloric caeca were fixed in the following fixatives: Bouin's, Helly's, 4% osmic acid in sea water, and 10% neutral formalin with post-chroming. Materials fixed in osmic acid were embedded in Epon, sectioned at a thickness of one micron on a Porter-Blum ultramicrotome, and stained with Richardson's (Richardson, Jarett, Fink, 1960) stain. This preparation is useful in demonstrating some details of cellular morphology, but it is poor for cytoplasmic inclusions. Materials fixed in other fixatives were embedded in paraffin and sectioned at 5 microns thickness. The combination of Helly's fixative and Mallory's phosphotungstic acid hematoxylin stain (PTAH) gave the best results in demonstrating cytoplasmic inclusions such as the secretory and storage granules. Altman's acid fuchsin and Heidenhain's iron hematoxylin also stained the secretory granules well. Polysaccharide compounds were defined by periodic acid-Schiff's (PAS) reagent. The mercuric bromphenol blue method of Mazia (Mazia, Brewer, Alfert, 1953) was used to demonstrate proteins. Materials fixed in neutral formalin and post-chroming were colored with sudan black to reveal lipid deposits.







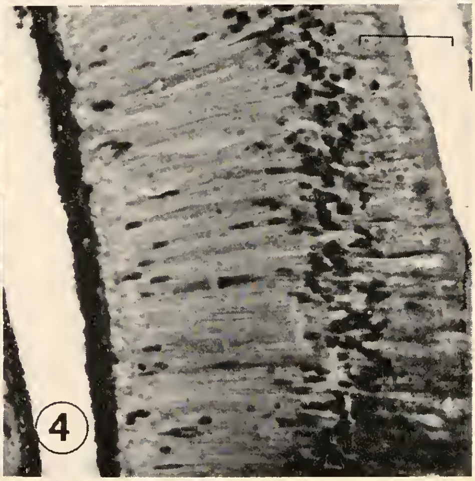


FIGURE 1. Side wall of the pyloric caecum in a feeding animal showing the general histology. Note the relationship of the secretory granules (s), the vacuole (v), the nucleus (n) in the zymogen cell. Note also the nucleus of storage cells (ns). Paraffin. Altman's acid fuchsin-hematoxylin. Scale: 25 μ.

FIGURE 2. Side wall of the pyloric caecum of a feeding animal (mesothelium is not present in this figure), showing the storage granules (g), secretory granules (s) and the vacuole (v). Paraffin. PTAH. Scale: 25 μ.

FIGURE 3. Cross section of the pyloric caecum showing the oral gutter (g) of the median duct where the epithelium proper is composed almost entirely of special current producing cells. Note the thickened middle layer (m) and the coagulated PAS-positive fluid (f) in the lumen

The starvation experiment was carried out at the Friday Harbor Laboratories in the summer months of 1965 and again in 1966. The animals were placed in a small aquarium with circulating sea water at 13–15° C. These animals appeared to be healthy and were able to right themselves even after ten weeks of starvation. Histological sections of the pyloric caeca were prepared from animals at 4-, 8- and 10-week intervals of starvation. A related species, *Leptasterias pusilla,* collected from Shell Beach, northern California, in April, 1966, was also examined. They were subjected to starvation in a recirculating sea water aquarium at 10° C, at Sacramento State College, Sacramento, California. These animals died in the 4th week but apparently not of starvation, as histological sections of the pyloric caecum showed no changes of nutrient reserves in this organ.

General Histology

The general organization and histology of the pyloric caeca in *Leptasterias* corresponds closely to that of *Asterias forbesi* which has been described in detail by Anderson (1953). The general structure is described here only briefly for comparative purposes, except for areas which reveal new or complementary information.

The wall of the pyloric caecum, as in all other starfishes, consists of three layers; an outer mesothelium, a middle layer of connective, muscular and nervous tissues and an inner digestive epithelium (Fig. 1).

The mesothelium or peritoneum is a layer of simple, flagellated, cuboidal epithelium. The thickness of this layer varies depending on the position or the state of contraction. It may be stretched and squamous-shaped, or crowded and low columnar-shaped. As low columnar cells, they measure 5–8 microns tall and a little less in width. The nucleus is oval in shape and it occupies most of the cell.

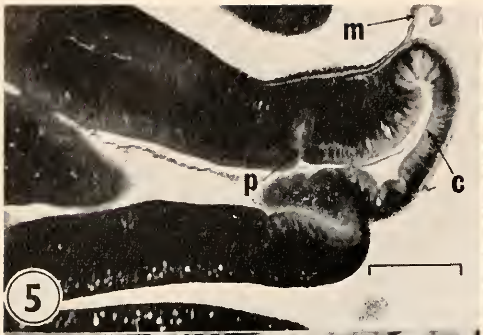
The middle layer of connective tissue, muscle fiber and nerve plexus again varies in thickness. It is thicker at the floor and roof of the median duct of the pyloric caecum where all three components are clearly shown, yet in other areas it may be so thin that only the nerve plexus and some connective fibers can be detected. The floor of the median duct is evaginated longitudinally to form a definite "gutter" (Fig. 3). The nerve plexus and muscle fibers are highly developed in this area, more so than at any other places in the pyloric caeca.

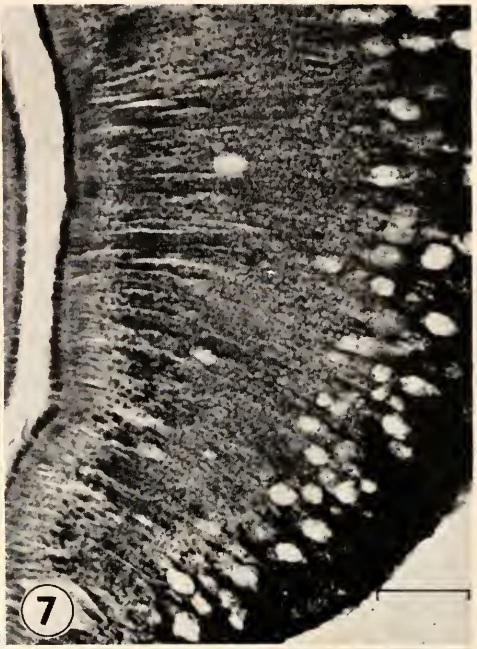
The digestive epithelium, as in that of *Asterias* (Anderson, 1953), consists of four cell types: (1) storage cells, (2) zymogen cells, (3) special current producers, and (4) mucous cells. All of the cells are cemented by terminal bars at the distal junctions (Fig. 6). In cross sections they all appear irregular or polygonal in shape except the vacuoles in the zymogen cell which are perfectly spherical (Figs. 8, 9).

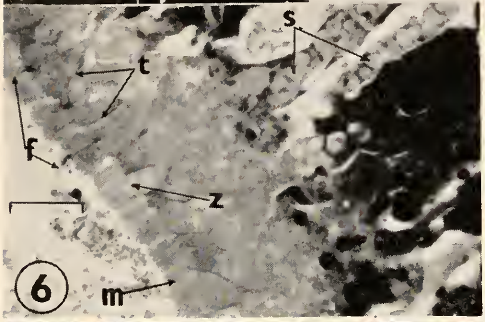
The storage cells are the major cell type of the digestive epithelium which line all the lumina of the pyloric caeca except the roof and oral gutter of the median duct. The cell measures 50 microns tall at the folds but reaches 120 microns in some other areas and it measures only 2 to 3 microns in diameter. The nuclei are

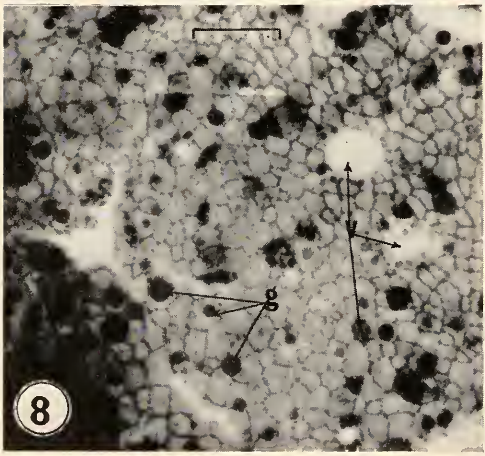
of the caecum. Note also the transitions (arrow) between the short special current producers and the tall storage and secretory cells. Paraffin. PAS. Scale: 100 μ.

FIGURE 4. Side wall of the pyloric caecum of a brooding animal (4 weeks along) showing that the zymogen granules and vacuoles have disappeared. This slide was prepared exactly as the one shown in Figure 1. Scale: 25 μ.









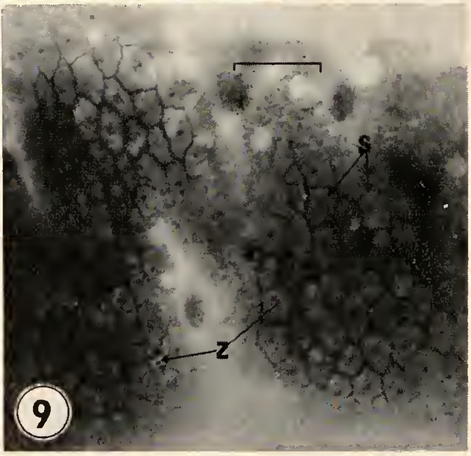


Figure 5. Cross section of the pyloric caecum of a feeding animal showing the roof of the median duct between the two mesenteries (m). Note the shorter special current producing cells (c) and the pit (p) in the side wall of storage and secretory area. Paraffin. PAS. Scale: 100 μ.

Figure 6. Apical ends of the digestive epithelium of a feeding animal showing the microvilli (m) of the brush border, flagellum (f), terminal bars (t), secretory granules (s), and the free end of the zymogen cell (z). Epon. Richardson's stain. Scale: 5 μ.

Figure 7. Side wall of the pyloric caecum of a feeding animal showing the lipid at the basal end of the digestive epithelium. Note also the vacuoles in zymogen cells. Paraffin. Sudan black, wet mount. Scale: 25 μ.

Figure 8. Tangential section at the lower middle zone of the digestive epithelium, showing the shape of the epithelial cells in cross section. Note the storage granules (g) in the storage cells and vacuoles (v) in zymogen cells. Paraffin. PTAH. Scale: 10 μ.

elongated and oval in shape and are located in the middle zone of the cells (Fig. 1). The apical ends of the storage cells are provided with a distinct brush border which consists of numerous microvilli (Fig. 6), adding further evidence to support their role as absorptive cells. Each cell also bears a flagellum with its basal body and rootlets centrally located in the apical end of the cell (Figs. 6, 9). This is different from that of *Asterias* in which the basal body is eccentric (Anderson, 1953). The functional significance of this difference is not understood. A number of greenish pigment granules, soluble in formalin, are also present in this region. The ground cytoplasm of the storage cells reacts strongly with PAS reagent except at the apical end where the pigment granules are located (Figs. 3, 5). The PAS reaction is not affected by digestion with diastase. Coarse storage granules are localized at the upper middle zone of the cells (Figs. 2, 8). These granules react positively with PAS, PTAH and mercuric bromphenol blue. The staining behavior thus suggests that the storage granule is a carbohydrate and protein complex. The sudanophilic materials (lipids) are mostly situated at the basal ends of the cells (Fig. 7).

The zymogen cells occur together with the storage cells but are less numerous. They can be identified by the presence of the secretory granules and clear vacuoles (Figs. 1, 2, 7, 8). The cell is flask-shaped with its greatest diameter at the vacuole and lacks the brush border and flagellum at the apical end (Fig. 7). The nuclei are oval or round and are less basophilic than those of the storage cells. They are located at the basal end immediately below the vacuoles (Fig. 1). The secretory granules are well preserved in Helly's fixative and neutral formalin but deformed or clumped together in materials fixed in Bouin's fluid. In most cases, they are arranged in rows between the vacuoles and the apical surface but sometimes they can be observed below the vacuoles or protruding among the brush borders of the adjacent storage cells (Fig. 6).

The special current producers are much shorter than the storage or zymogen cells. They measure 40 to 50 microns in length and line primarily the roof and floor of the median duct (Figs. 3, 5). They have elongated nuclei close to the basal end of the cell and at the apical ends, the brush border and flagella are most prominent. Judging from their highly developed brush borders, it is hard to conceive that they function only as current producers. It is likely that they also serve for absorptive purposes.

A few mucous cells are dispersed among other cells in all parts of the caeca but are more abundant among the current producing cells. The nuclei are round, less basophilic and basally located. The mucous cells, as in zymogen cells, lack both brush border and flagella.

Changes during Brooding and Starvation

The major changes in the pyloric caeca during both brooding and starvation occur primarily in the storage and zymogen cells. Mucous cells and the special current producers do not appear to be affected.

After four weeks of brooding, histological sections show that lipids, and storage

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Figure 9. Tangential section at the apical end of the digestive epithelium, showing the storage cells (s) with the centrally located basal bodies of the flagella and the zymogen cells (z) with their secretory granules. Paraffin. PTAH. Scale: $10\ \mu$.

granules, have disappeared from the storage cells. The secretory granules and vacuoles in the zymogen cells have also become inconspicuous (Fig. 4). It is not possible to assess the changes of the nutrient material in the storage cells in quantitative terms, but the changes in zymogen cells can be expressed in a more precise manner because the zymogen cells are identified by their clear vacuoles and during brooding the number of vacuoles decreases. Thus, by comparing the numbers of vacuoles per unit area in the pyloric caeca between feeding and brooding animals, one can get a relatively clear picture. It is estimated that after four weeks of brooding the zymogen cells decrease by about 80%. This does not mean, however, a decline of the cell population; it rather indicates the inactivation of secretory activity. Ten weeks after brooding, which is the end of brooding activity, all observed cellular inclusions in the storage and zymogen cells have been depleted. The ground cytoplasm of the storage cells no longer reacts with PAS reagent. In fact, there are signs of structural breakdown at the apical ends of the digestive epithelium.

In the starved animals there is little or no change in cellular inclusions in both storage and zymogen cells after four weeks of starvation. Even after 10 weeks, there are still some lipids and the storage granules are plentiful. The ground cytoplasm still reacts with PAS reagent although less intensively. In the zymogen cells both the secretory granules and vacuoles are still obvious. This result differs from that of *Asterias* in which all the nutrients are depleted after 8 weeks starvation (Anderson, 1953). Anderson surmised that 8 weeks is about the maximum length of time during which the animal can survive without feeding. *Leptasterias* can apparently survive much longer and the same is true in *Pisaster* which can last as long as 48 weeks without feeding (Mauzey, 1967). This difference in the maximum starvation periods among the three species is likely to be due to the time of year during which the experiments were made; it is well documented that the nutritional level and metabolic rate in the pyloric caeca vary in an inverse relationship with those of the gonad at different seasons of the year (see reviews by Anderson, 1966; Boolootian, 1966). Other factors such as the age and size of the animal may also be important. Finally, there just may be significant differences between species.

Discussion

The most impressive feature of the digestive epithelium is its great height, particularly that of the storage and secretory cells. The tallest cells reach 120 microns with a diameter of only 2 to 3 microns. Thus the ratio between height and diameter is of the order of about 50. Because of the extreme height there is a definite functional zonation or polarity in the cell along its long axis. In the storage cells the nutrient and other cellular inclusions are arranged from the apical end downwards into three bands: pigment granules, storage granules and lipid deposits. In the zymogen cells, the secretory granules most frequently take a position between the vacuole and the apical surface. Because of this relationship, any change of the shape, size or position of the vacuoles would inevitably be connected with the movement of the secretory granules. Therefore, the vacuoles may operate as vehicles to transport the zymogen granules into the lumen of the pyloric caecum.

The highly developed muscular tissue in the oral gutter of the median duct is significant when considering the function of this specialized area. In some particle-feeding starfishes, the whole floor of the median duct is developed into a Tiedemann's pouch which has been designated as a "flagellary pumping organ" by Anderson (1960, 1962, 1966). An examination of the floor of Tiedemann's pouch in *Henricia sanguinolenta* has revealed most elaborate circular and longitudinal muscular elements (Fig. 10). There is little doubt that this tissue is directly involved in the pumping mechanism; thus, the transport of nutrient media in the pyloric caeca is accomplished by both flagellary movement and muscular contraction.

The utilization of food reserves during brooding in *Leptasterias* is apparently much greater than during starvation. For example, most of the detectable nutrient in the pyloric caeca is depleted after 4 weeks of brooding, but there are still plenty of nutrients left even after ten weeks of starvation. It is likely that the nutrients in the pyloric caeca can only support the first phase of brooding and at the latter part of brooding, nutrients from other sources, such as the body wall, must be utilized.

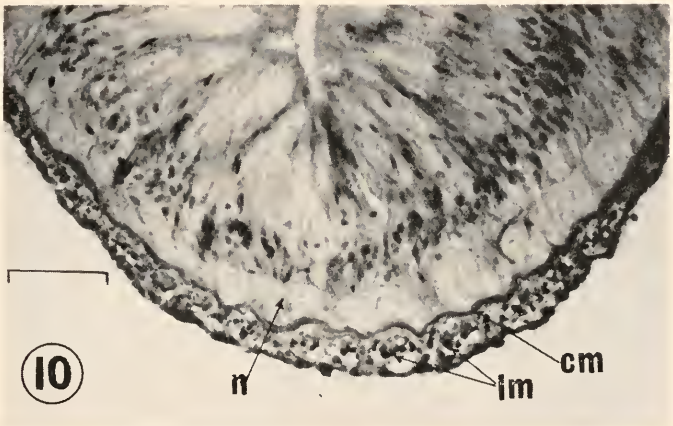

FIGURE 10. Cross section of the oral gutter of Tiedemann's pouch in the starfish, *Henricia sanguinolenta* showing the highly developed nerve plexus (n), the circular (cm) and longitudinal muscles (lm) at the floor of the gutter. Paraffin. PTAH. Scale: 40 μ.

The inactivation of the zymogen cells in the pyloric caeca during brooding provides circumstantial evidence of why *L. hexactis* stops feeding during brooding and how the embryos of *L. groenlandica* survive in the cardiac stomach. It is not certain, however, if the zymogen cells are inactivated after the nutrient has been depleted by starvation. In *Asterias* the depletion of nutrient by starvation has no effect on the zymogen cells (Anderson, 1953), but in *Pisaster* both the nutrient and zymogen granules disappear from the pyloric caeca during the time when the feeding frequency is low and the pyloric caeca are small, or after a long period of starvation (Mauzey, 1966, 1967). As it has been pointed out by Anderson (1966) the so-called zymogen cells may in fact represent several kinds of secretory cells and there is still no direct evidence of the enzymatic nature of the secretions. Wilson and Falkmer (1966) have found insulin-producing cells in the pyloric caeca of *Pisaster* and by using the histochemical methods of Kvistberg (Kvistberg, Lester and Lazarow, 1966) I have identified insulin-producing cells in the pyloric caeca of *Henricia sanguinolenta* (unpublished data). These cells are otherwise inseparable from zymogen cells, except in *Henricia* where the presumed insulin granules are smaller than the zymogen granules, and most of the insulin-producing cells are located at the lateral diverticular instead of the roof of the median duct where most of the zymogen cells are found. Thus, the pyloric caeca may also function as an endocrine organ.

SUMMARY

1. The general histology of pyloric caeca in *Leptasterias hexactis* is similar to that of *Asterias forbesi,* which is already known.

2. Food reserves such as lipid, polysaccharide and storage granules of carbohydrate-protein complex, are abundant in the pyloric caeca of feeding animals, but disappear after four weeks of brooding. Nutrient reserve from other sources is probably utilized during the last phase of brooding.

3. Accompanying the depletion of nutrient material during brooding, zymogen cells are also inactivated; this is regarded as evidence of why the animals stop to feed during brooding and how the embryos can survive in the cardiac stomach as in the case of *Leptasterias groenlandica.*

4. In *L. hexactis* which have been starved for 10 weeks, the nutrient reserves are still plentiful in the pyloric caeca and, up to this stage, there is little or no detectable change in the zymogen cells.

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SURVIVAL AND GROWTH OF LARVAE OF THE EUROPEAN OYSTER (*OSTREA EDULIS* L.) AT DIFFERENT TEMPERATURES

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A small number of European oysters (*Ostrea edulis*) was imported into the United States in 1949 for a study of the adaptability of this species to our waters. It was hoped that this oyster might be suitable for colder areas because in its northern range the European oyster reproduces at temperatures too low for the American oyster (*Crassostrea virginica*) to propagate (Loosanoff, 1955; Loosanoff and Davis, 1963). Some of these oysters were kept at Milford where we have reared a new generation almost every year to replenish our stock and to supply seed to other areas for studies of survival and growth. Others were planted in several estuarine areas of Maine; they reproduced naturally and have become established in Boothbay Harbor (Loosanoff, 1955). Seed oysters reared at Milford have been shipped to interested state shellfish biologists in California, Washington, and Alaska, but at present we know of no area in these states where a population has become established. Several single individuals have been found, however, attached to dead shells of Japanese oysters in Tomales Bay, California, where large numbers of Milford-reared *O. edulis* have been used in transplanting experiments (Loosanoff, personal communication).

In recent years shellfish hatcheries have been used more and more to propagate desirable commercial mollusks in areas where they do not propagate naturally or where naturally produced seed is insufficient. Successful operation of these hatcheries depends upon an adequate knowledge of the spawning habits of the mollusks and of the environmental requirements of their larvae.

Walne (1956, 1963, 1964, and 1965) studied several aspects of the rearing of larvae of *O. edulis,* especially the food requirements, and Davis and Ansell (1962) studied the salinity tolerance of these larvae. Korringa (1941) reviewed many field studies in an attempt to correlate temperature with duration of the larval period of *O. edulis* in European waters, and he believed that the length of the free-swimming period depended primarily on temperature. Walne (1965) reported on the influence of food supply and temperature on the growth of *O. edulis* larvae, but did not suggest an optimum temperature nor an upper limit of the temperature range for growth and survival. He suggested a "biological zero temperature of 13° C" (p. 30) at which no growth should occur. From his determinations of 24-hour growth rates he concluded that, "In the pelagic phase the time taken to grow from 175–250 μ decreases from 14 days at 17° C to 5 days at 25° C" (p. 42). No previous work, however, has been done on the survival and rate of growth of these larvae from time of release to setting under controlled conditions at different constant temperatures. In the present study we determined the temperature range within which the larvae of *O. edulis* can exist and the optimum temperature for their growth.

METHODS

A series of 11 experiments was conducted in which duplicate 1-liter cultures of larvae were grown at each of six different temperatures in each experiment.

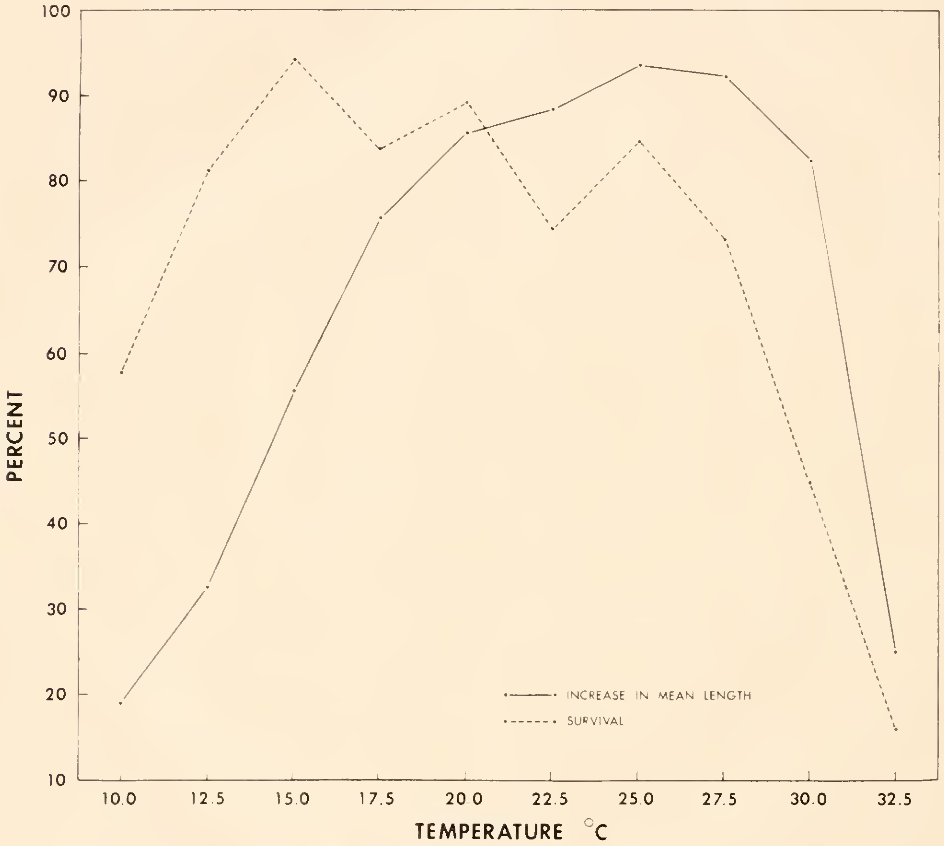

FIGURE 1. Survival and growth of larvae of *O. edulis* at different temperatures. Growth is expressed as average percentage increase in mean length for 11 experiments (Table I). Survival is average percentage survival in 11 experiments (Table II).

Since our apparatus can maintain only six different constant temperatures at a time, it was necessary to use a different series of temperatures in different experiments to cover the range from 10° to 32.5° C in 2.5° C steps. We considered it desirable to repeat certain reference temperatures in all experiments to facilitate comparisons among experiments. The temperatures of 20° and 27.5° C, therefore, were included in all experiments.

An accurately determined number, usually between 6000 to 8000, of recently released larvae was placed in each 1-liter polypropylene beaker containing filtered, ultraviolet-treated sea water (salinity 27 ± 0.5 ppt). All larvae were fed daily with a mixture of *Monochrysis lutheri, Dicrateria* sp. BII, and *Chlorella* sp. (Indiana University Collection #580). The sea water in each culture was changed every second day to eliminate waste products of larval metabolism. In most experiments 33 mg/l of Sulmet were added to each culture with each change of water to control bacteria which were troublesome at the higher temperatures. [Sulmet, sodium sulfamethazine, is a trade name of American Cyanamid Co. Mention of trade names does not imply endorsement of the product by the Bureau of Commercial Fisheries.]

TABLE I

Growth of O. edulis *larvae at different temperatures**

Expt. no.	Temperature (°C)									
	10.0	12.5	15.0	17.5	20.0	22.5	25.0	27.5	30.0	32.5
1	36.5	53.9	—	78.6	100.0	—	—	96.2	—	—
2	21.6	39.6	—	74.6	89.7	—	—	100.0	—	—
3	1.8	14.1	46.4	89.4	100.0	—	—	38.4	—	—
4	16.1	39.5	64.8	95.0	100.0	—	—	91.0	—	—
5	—	—	—	—	79.4	95.6	99.7	100.0	97.1	32.5
6	—	—	—	—	85.3	90.4	100.0	89.9	73.8	17.6
7	—	33.3	—	—	62.6	78.6	89.1	100.0	76.6	—
8	—	40.6	76.9	82.3	86.1	—	96.5	100.0	—	—
9	—	19.6	44.9	63.1	80.3	—	89.4	100.0	—	—
10	—	39.4	61.1	65.4	78.8	—	94.7	100.0	—	—
11	—	13.4	38.5	57.7	81.5	—	86.1	100.0	—	—
Average	19.0	32.6	55.4	75.8	85.8	88.2	93.6	92.3	82.5	25.1

* Increase in mean length is given as percentage of greatest increase at any temperature within that experiment. Figures are averages for duplicate cultures at each temperature in each experiment.

To determine the percentage survival and increase in mean length, the experiments were terminated after 8 or 10 days at the experimental conditions. Larvae in those cultures at near optimum temperatures were setting between the 10th and 12th days and satisfactory samples could no longer be obtained because once setting begins, not all of the larvae can be collected on the screens and resuspended. The larvae from each beaker were transferred to 250 ml of sea water in a graduated cylinder. The contents of the cylinder were thoroughly stirred to insure uniform distribution of the suspended larvae and a 4-ml quantitative sample (1.6% of the total population) was withdrawn and preserved with formalin. We examined each sample under a compound microscope, determined the number of larvae that survived the treatment and measured a random group of 50 individuals. Unpublished data indicate that this sampling technique yields survival figures accurate only to about ±10%.

No one set of conditions can be considered "control" conditions in these experiments; therefore, survival at each temperature is expressed as a percentage of the number of larvae surviving in the pair of cultures, at a single temperature, that had the highest survival within that experiment. Increase in mean length is likewise expressed as a percentage of that of larvae in paired cultures showing the greatest increase. Since temperatures of 20° and 27.5° C were employed in each experiment, a comparison of percentages in successive experiments seems justifiable. All larvae were from *O. edulis* stock maintained at Milford except those used in Experiment 11. For this experiment we used larvae of *O. edulis* from the group that had become established in Boothbay Harbor, Maine.

TABLE II

Survival of O. edulis *larvae at different temperatures**

Expt. no.	Temperature (°C)									
	10.0	12.5	15.0	17.5	20.0	22.5	25.0	27.5	30.0	32.5
1	66.0	60.9	—	100.0	71.0	—	—	81.2	—	—
2	67.3	85.6	—	87.5	100.0	—	—	67.3	—	—
3	44.6	75.0	100.0	54.5	91.0	—	—	49.0	—	—
4	53.0	87.6	91.2	100.0	96.9	—	—	2.8	—	—
5	—	—	—	—	96.0	79.6	100.0	77.8	71.9	23.4
6	—	—	—	—	85.4	74.0	87.4	100.0	1.6	8.5
7	—	100.0	—	—	65.2	69.7	64.8	72.2	61.1	—
8	—	90.0	93.5	100.0	94.4	—	92.9	96.3	—	—
9	—	54.3	95.7	71.5	100.0	—	68.0	73.4	—	—
10	—	89.5	91.0	84.5	91.4	—	100.0	85.2	—	—
11	—	88.4	94.1	71.5	90.4	—	80.5	100.0	—	—
Average	57.7	81.2	94.3	83.7	89.2	74.4	84.8	73.2	44.9	16.0

* Survival is given as percentage of number of larvae surviving in the best culture within that experiment. Figures are averages for duplicate cultures at each temperature in each experiment.

EFFECT OF TEMPERATURE ON GROWTH

The average rate of growth of *O. edulis* larvae increased progressively as the temperature increased from 10° to 25° or 27.5° C and then decreased at 30° and 32.5° C (Fig. 1 and Table I). Although some growth did occur at 10° and 12.5° C and some larvae reached setting size at 12.5° and 15° C, we obtained no spat in these experiments at temperatures below 17.5° C. Growth was satisfactory (70% or more of maximum) only within the range from 17.5° to 30° C; at temperatures above and below this range, growth was too slow to be practical for shellfish culture. In nature a prolonged larval period would cause excessive losses due to predation and dispersal and in hatcheries it would greatly increase the cost of labor and make for inefficient use of equipment.

No one temperature consistently gave the most rapid growth of the larvae, although 27.5° C was optimum for growth in 7 of the 11 experiments (Table I). Moreover, if we disregard experiments in which survival was slightly less than 50% at 27.5° C (Table II), the average increase in mean length at 27.5° C becomes 96.8%, higher than the average for any other temperature. From examination of the living cultures, also, it was clear that the larvae grew best at 27.5° C. In some experiments, however, the proliferation of toxin-producing bacteria (or, as in Experiment 4, what appeared to be pathogenic bacteria) at 27.5° C and higher temperatures caused slow growth and poor survival.

Effect of Temperature on Survival

Survival was much more erratic than growth (Fig. 1 and Table II). Except for cultures kept at 10° C and those at 30° and 32.5° C, the average survival at each temperature was within the acceptable range (70% or more of optimum). Even at 10° and 30° C survival was poor, perhaps, largely because these unfavorable temperatures weakened the larvae, thus leaving them more susceptible to bacterial toxins and diseases. Increased bacterial populations, particularly noticeable at 30° and 32.5° C, could not be controlled by Sulmet. Nevertheless, the greatly reduced rate of growth at 32.5° C suggests that this temperature affected these larvae directly.

The excellent survival at 27.5° C in Experiments 6, 8, and 11 (Table II) confirmed our impression that poor survival at this temperature in other experiments was not the direct effect of temperature on the larvae but rather an indirect effect of the rapid proliferation of bacteria, which could not be adequately controlled by Sulmet. In many of the experiments the mortality at temperatures of 27.5° and 30° C occurred after the larvae were almost at setting size. In no experiment did we get as many larvae to set at 27.5° C as we did at somewhat lower temperatures. In Experiments 5 and 6, for example, starting with the same number of larvae at each temperature we obtained a total of 4964 spat at 20° C, 4863 at 22.5° C, 3709 at 25° C, 602 at 27.5° C, and only 14 at 30° C.

Setting times at each temperature varied considerably in these experiments because of variations in the quality of food cultures. Walne (1965) also reported that the rate of growth of *O. edulis* larvae at any given temperature varied in proportion to the quantity of food supplied, as well as the kind of algae used as food. In these experiments approximate setting times were as follows: 17.5° C—26 days, 20° C—14 days, and at 25°, 27.5°, and 30° C beginning of setting varied from the 8th to the 12th days.

Survival and Growth of Spat

A single experiment was conducted to determine the survival and rate of growth of *O. edulis* spat at different temperatures. Spat that had set less than 48 hours previously were placed at 10°, 12.5°, 15°, 17.5°, 20°, and 27.5° C. Survival was good at all temperatures except 10° C where growth was not appreciable; the spat still averaged only 0.3 mm in diameter after 30 days. Spat kept at 12.5°, 15°, 17.5°, 20°, and 27.5° C for 30 days averaged 0.5, 0.7, 1.5, 1.9, and 2.5 mm, respectively.

Evaluation and Recommendations for Culture

As shown previously, the maximum and minimum temperatures for growth of bivalve larvae depend to a large degree on the type of food provided and on salinity

(Davis and Calabrese, 1964). Although we believe the mixture of flagellates and *Chlorella sp.* (580) used as food in these experiments probably suffices at both temperature extremes, we cannot preclude the possibility that with other foods *O. edulis* larvae might grow satisfactorily at even lower or higher temperatures than are indicated in these experiments. Salinity (approximately 27‰) was about optimum (Davis and Ansell, 1962).

The temperature range for satisfactory growth of *O. edulis* larvae (17.5° to 30° C) is further evidence of the adaptation of this species of oysters to lower temperatures than *C. virginica,* since larvae of the latter species grow satisfactorily under our experimental conditions only at temperatures above 22.5° C (Davis and Calabrese, 1964). This difference in temperature tolerance of the larvae of *O. edulis* has been maintained even after approximately 15 generations grown at Milford. Moreover, the tolerance of larvae released by the stock of *O. edulis* that had become established at Boothbay Harbor, Maine (Experiment 11) did not differ from that of the larvae of Milford parents, although these two populations of *O. edulis* have been separated since 1949.

Walne (1965) reported that a brood of larvae reared at 14.3° C began setting on the 49th day (p. 29). Although 17.5° C was the lowest temperature at which we actually obtained setting in our experiments, larvae were grown to setting size at 12.5° and 15° C and we believe that setting could be obtained at 12.5° C if an experiment were continued long enough.

Walne (1965) stated that at all food-cell densities tested the uptake at 20° C is about 70% of that at the same cell density at 24° C (p. 34). Our data show that the average increase in mean length at 20° C is approximately 91% of that at 25° C. If we assume that the volume increases as the cube of the increase in mean length, then the increase in volume at 20° C is about 75% of that at 25° C or in reasonably close agreement with Walne's figure on food uptake.

It might be practical in hatchery operations to rear *O. edulis* larvae at 27.5° C until they reach a length of about 250 to 275 μ to take advantage of the more rapid growth and then reduce the temperature to 20° or 25° C to obtain a higher percentage of spat. Growth at 27.5° C, however, was not sufficiently faster than at 25° C to warrant the increased risk. Usually, it required only 1 or 2 days longer to obtain spat at 25° C than at 27.5° C and sometimes setting began simultaneously at both temperatures.

SUMMARY

1. The temperature range for satisfactory growth of *O. edulis* larvae (70% or more of optimum) was from 17.5° to 30° C.

2. The temperature range for satisfactory survival (70% or more of optimum) was from 12.5° to 27.5° C. Even at 10° and 30° C survival was poor, perhaps, because the unfavorable temperatures weakened the larvae, making them more susceptible to bacterial toxins and diseases.

3. In these experiments approximate setting times were as follows: 17.5° C—26 days, 20° C—14 days, and at 25°, 27.5°, and 30° C beginning of setting varied from the 8th to the 12th days.

4. More spat were obtained at 20° to 22.5° C than at higher temperatures.

5. It is suggested that larvae be reared to setting size at temperatures from 25° to 27.5° C, then kept at 20° to 22.5° C during setting to obtain fastest growth of larvae and highest percentage setting.

6. Spat kept at 10° C showed virtually no growth; at temperatures from 12.5° to 27.5° C growth of spat increased with each increase in temperature.

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A COMPARATIVE STUDY OF LEUCOPHORE–ACTIVATING SUBSTANCES FROM THE EYESTALKS OF TWO CRUSTACEANS, *PALAEMONETES VULGARIS* AND *UCA PUGILATOR*[1]

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After the discovery by Koller (1925) that crustacean chromatophores are under endocrine control, a number of investigators found that neuroendocrine tissues from one species of crustacean would activate the chromatophores of another (Fingerman, 1963). For example, Brown and Scudamore (1940) demonstrated that extracts of sinus glands from the prawn, *Palaemonetes vulgaris,* and the fiddler crab, *Uca pugilator,* concentrate the red pigment of the prawn and disperse the melanin of the crab, and that these effects are due to different substances. More recently Fingerman and Couch (1967) showed that although both *Uca* and *Palaemonetes* possess substances that disperse the melanin in *Uca,* disperse the red pigment in both species, and concentrate the red pigment in both species, the substance present in an extract prepared from organs of either species that is effective in either concentrating or dispersing the red pigment of the prawn is different from the substances in the same extract which has the same qualitative effect on the red pigment in the crab.

Much less information, however, is available about leucophores of crustaceans than about the black, brown-black, and red chromatophores. For example, no one has previously determined whether extracts of eyestalks from *Palaemonetes vulgaris* have any effect on the leucophores of *Uca pugilator.* In fact, only recently has evidence been presented for a white pigment-dispersing substance in *Uca pugilator* (Rao, Fingerman and Bartell, 1967). Earlier Sandeen (1950) had reported the presence of a white pigment-concentrating substance in *Uca pugilator.* Brown (1935) found that extracts of eyestalks from *Palaemonetes* cause concentration of its white chromatophoric pigment. However, no evidence is available for the presence of a white pigment-dispersing substance in this genus. In view of the paucity of information about leucophores of crustaceans the present investigation was designed (1) to determine the effect of extracts of eyestalks from *Palaemonetes vulgaris* on the leucophores of *Uca pugilator,* (2) to compare the leucophore-activating substances present in the eyestalks of *Uca* and *Palaemonetes* by means of their behavior during gel filtration, and (3) to obtain further information concerning the antagonism described by Rao, Fingerman and Bartell (1967) between the white pigment-dispersing and -concentrating substances from *Uca.*

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Materials and Methods

The specimens of *Palaemonetes vulgaris* and some of the *Uca pugilator* used in this investigation were collected in the vicinity of Woods Hole, Massachusetts. The remainder of the *Uca pugilator* were from the area of Panacea, Florida. We thank the members of the Supply Department at the Marine Biological Laboratory and the personnel of the Gulf Specimen Company, Panacea, Florida, for their cooperation.

In order to determine the magnitude of the response to a chromatophorotropin the chromatophores were staged until the response had been completed by using the system of Hogben and Slome (1931) in which Stage 1 represents maximal pigment concentration, Stage 5 maximal dispersion, and Stages 2, 3, and 4 the intermediate conditions. The chromatophorotropic activity of the extract could then be expressed in terms of the Standard Integrated Response as defined by Fingerman, Rao and Bartell (1967) which is a measure of both the amplitude and duration of the response.

Extracts were prepared in three different ways from eyestalks of *Uca* as well as *Palaemonetes.* Method I was used for preparing fresh aqueous extracts of the eyestalks. The eyestalks, dissected fresh from the donors, were placed in an embryological dish, triturated with a glass rod, and extracted with the desired volume of either crustacean saline (Pantin, 1934) or distilled water. The extract was centrifuged at 1500 × *g* and at room temperature for 10 minutes and the supernatant was then used in the experiments. The extracts prepared directly in saline were used for immediate determination of the chromatophorotropic activity of the extracted tissue whereas the extracts prepared directly in distilled water were used in column chromatographic experiments. Method II was used for preparing freeze-dried extracts of the eyestalks. Freshly dissected eyestalks were first extracted in the particular desired volume of distilled water as noted below where the appropriate experiments are described. The extract was then centrifuged at 1500 × *g* and at room temperature for 10 minutes, and the supernatant was subsequently lyophilized. The freeze-dried material was dissolved in crustacean saline for assay. Method III was used for preparing the ethanol-soluble fraction of the eyestalks. Freshly dissected eyestalks were blotted with a paper towel, then placed in an embryological watch glass, triturated, and extracted in 10 ml of ethanol taking care to avoid excessive stirring during the extraction. The extract was centrifuged at 1500 × *g* for 10 minutes and the supernatant was decanted into an evaporating dish. The alcohol was then allowed to evaporate at room temperature and the remaining material was eluted in saline for immediate assay or in distilled water or ethanol for use in column chromatographic experiments.

Specimens of *Uca pugilator* were used in the assays for the white pigment-dispersing and -concentrating substances. Eyestalkless crabs, their white pigment is maximally dispersed, were used in the assays for white pigment-concentrating substances, whereas intact fiddler crabs adapted to a black background, their white pigment is maximally concentrated, were used in the assays for white pigment-dispersing substances. In the latter assay only crabs from Panacea were used because they are capable of concentrating their white pigment maximally when adapted to a black background (Rao, Fingerman and Bartell, 1967). In contrast, as Brown and Sandeen (1948) had reported earlier, fiddler crabs from Woods

Hole do not concentrate their white pigment to the maximum extent when adapted to a black background. During the period of assay the crabs were exposed to light intensities of 2.9–3.3 meter-candles and temperatures of 22–24° C. The number of animals used in each assay varied with the experiment and, therefore, will be given at the appropriate places below. Each extract that was assayed was injected in a dose of 0.05 ml per crab.

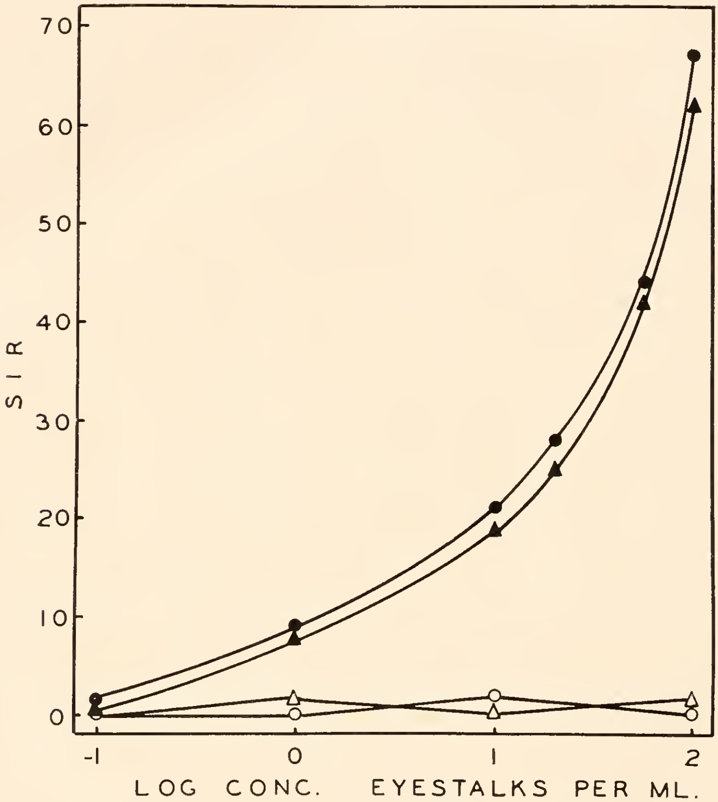

FIGURE 1. Relationships between the Standard Integrated Response (SIR) of the leucophores in *Uca pugilator* and the logarithm of the relative concentration of extracts of the eyestalks from *Uca pugilator*. White pigment-dispersing responses evoked by the extract prepared directly in saline (dots) and the ethanol-soluble fraction (solid triangles); white pigment-concentrating responses evoked by the extract prepared directly in saline (circles) and the ethanol-soluble fraction (empty triangles).

Two types of gels, Bio-Gel P-6 (Calbiochem) and Sephadex LH-20 (Pharmacia Fine Chemicals), were used in the present study for chromatographic separation of leucophore-activating substances. The size of the Bio-Gel column was 27 × 1.5 cm. The void volume of the column, as determined by filtering Blue Dextran 2000 (Pharmacia Fine Chemicals) through the column, was 14.0 ml. The material to be separated on the Bio-Gel was prepared in a small volume of distilled water (0.2 to 0.4 ml) and applied to the top of the column. Distilled water was used as the solvent. Two ml fractions were collected. Before being assayed each fraction was made isosmotic to the blood of the fiddler crab by adding one part of 400% crustacean saline to three parts of the eluted fraction. Each fraction was assayed on three eyestalkless crabs and three crabs adapted to a black background.

Sephadex LH-20 was equilibrated with ethanol which was used as the solvent. The size of the column was 28 × 1.5 cm, the void volume of the column was 21 ml. The ethanol extract (sample size 0.2 to 0.4 ml) was applied to the top of the column and then the solvent was allowed to flow through. Two ml fractions were collected. The alcohol in each sample was allowed to evaporate at room temperature after which the material in each sample was dissolved in 0.5 ml of crustacean saline and assayed on three eyestalkless *Uca* and three intact *Uca* adapted to a black background.

EXPERIMENTS AND RESULTS

The aim of the first series of experiments was to determine and compare the relationships between the standard integrated pigment-dispersing and -concentrating responses of leucophores in *Uca pugilator* and the concentration of eyestalk extracts prepared according to Methods I and III from the prawn and the crab. The fresh saline extract of crab eyestalks was prepared from 100 eyestalks dissected one each from 100 *Uca* and extracted in 1 ml of saline. The ethanol-soluble fraction was prepared from the contralateral eyestalks from the same 100 crabs. The ethanol-soluble fraction was likewise eluted in 1 ml of saline. A series of six dilutions from 100 to 0.1 eyestalks per ml was prepared from each of these extracts by using saline as the diluent. In similar fashion a saline extract and the ethanol-soluble fraction were prepared with eyestalks of *Palaemonetes*. In each experiment the extract of a given concentration was assayed on five eyestalkless *Uca* and five intact *Uca* adapted to a black background. Each experiment was repeated once. The averaged results shown in Figures 1 and 2 reveal that for both species the extract prepared directly in saline and the ethanol-soluble fraction of the eyestalks evoked nearly identical white pigment-dispersing activities at all concentrations tested. The aqueous extracts of eyestalks from *Uca* and *Palaemonetes*, and the ethanol-soluble fraction of the eyestalks from *Uca* failed to concentrate the initially dispersed white pigment in the chromatophores of *Uca*. However, the ethanol-soluble fraction of the eyestalks from *Palaemonetes* evoked white pigment concentration in *Uca*. The latter response was highest at the lowest concentration of the extract tested and decreased with increase in the concentration of the extract (Fig. 2).

The second series of the experiments was conducted to determine whether the technique of gel filtration would be helpful in determining whether the white pigment-dispersing substances from the prawn and the crab are identical or not. Fifty eyestalks, freshly dissected from *Uca*, were extracted in 0.4 ml of distilled water. After centrifugation the supernatant was fractionated on a column of Bio-Gel P-6. The white pigment-dispersing activity appeared at two places, fractions 7 and 12 (Fig. 3). The activity of fraction 12 is eight to ninefold higher than that of fraction 7. No white pigment concentration was detected. Similar results were obtained when distilled water extracts of 50 eyestalks from *Palae-*

monetes, and a mixture of the distilled water extracts from 25 eyestalks of *Uca* and 25 eyestalks of *Palaemonetes* were chromatographed on the column of Bio-Gel P-6 (Fig. 3). The above experiments were repeated twice with consistent results. On the basis of these experiments there is no reason to consider the white pigment-dispersing substances from the prawn and the crab are anything but identical.

In the next experiment the ethanol-soluble fraction of 50 freshly dissected eyestalks from *Uca* was prepared taking care to avoid excessive stirring during the extraction. After the alcohol had evaporated the material was eluted in 0.4 ml distilled water and subjected to chromatography on the Bio-Gel column. The white pigment-dispersing activity appeared at one place, fraction 7 (Fig. 4). This fraction also had most of the red pigment which is always extracted from the eyestalks along with the chromatophorotropins. The substances in fraction 7 were excluded from this gel which has an exclusion limit of 4600 daltons. No white pigment concentration was observed in this experiment also. Similar results were obtained when a distilled water eluate of the ethanol-soluble fraction from 50 eyestalks of *Palaemonetes* was chromatographed (Fig. 4).

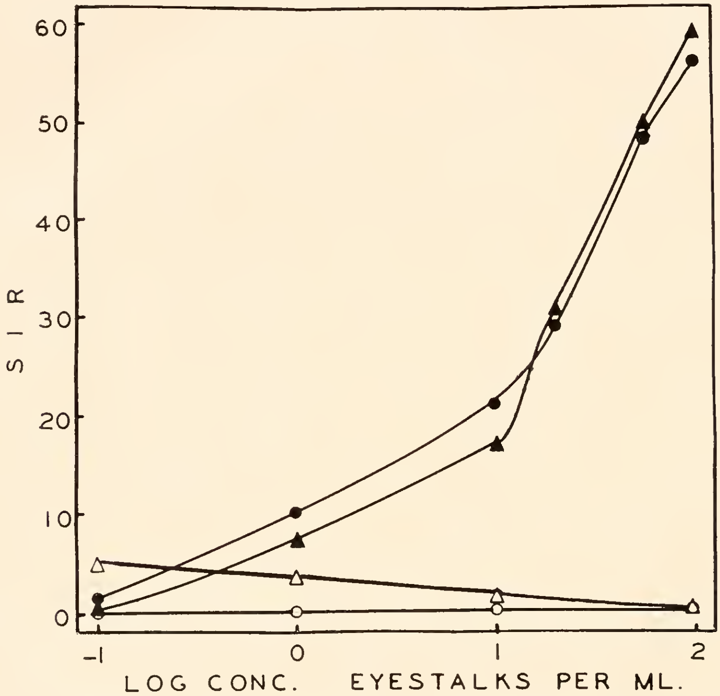

FIGURE 2. Relationships between the Standard Integrated Response (SIR) of the leucophores in *Uca pugilator* and the logarithm of the relative concentration of extracts of the eyestalks from *Palaemonetes vulgaris.* See Figure 1 for key to symbols.

Previous investigation (Bartell, Rao and Fingerman, 1967) revealed that when aqueous and alcohol extracts of eyestalks from *Uca* were filtered through Bio-Gel P-6 the melanin-dispersing activity appeared at two peaks, one in the void volume and the other at an R_f of 0.6. The first peak appeared to be due to the melanin-dispersing substance complexed with a lipid-containing material of high molecular weight and the R_f 0.6 peak of free peptide. By merely stirring the extract it was possible to split the complex and free the smaller component. To determine whether a similar complex accounts for the white pigment-dispersing activity recovered in the void volume of the Bio-Gel column the following experiment was performed.

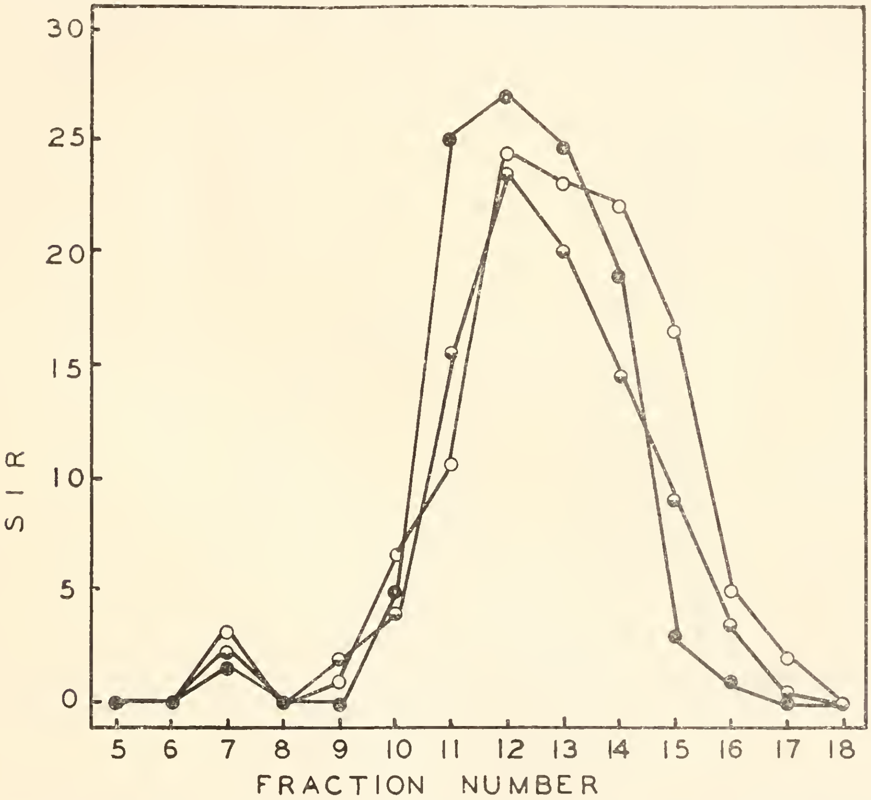

FIGURE 3. The white pigment-dispersing Standard Integrated Responses (SIR) evoked by the fractions of extracts of eyestalks prepared directly in distilled water from *Uca* (dots) and *Palaemonetes* (circles), and of the mixture of extracts of the eyestalks from *Uca* and *Palaemonetes* (half-filled circles) after filtration through a 27 × 1.5 cm column of Bio-Gel P-6. Flow rate: 48 ml per hour. Before assay the samples were made isosmotic to the blood of the fiddler crab. See text for details.

Fifty eyestalks of *Uca* were extracted in ethanol and during the process of extraction the extract was vigorously stirred with a glass rod. After the alcohol had evaporated from the ethanol-soluble fraction the material was eluted in 0.4 ml distilled water and chromatographed on Bio-Gel P-6. Using the same procedure an extract was made from 50 eyestalks of *Palaemonetes* and likewise chromatographed. The results shown in Fig 4 reveal that the white pigment-dispersing activity appeared at two peaks, fractions 7 and 12. These results indicate that the white pigment-dispersing substance in the ethanol fraction prepared without vigorous stirring is indeed also loosely bound to a heavier substance. The next set of experiments was designed to determine the chromatographic behavior of the white pigment-dispersing substance in the ethanol-soluble fraction prepared from eyestalks pretreated with 10 ml acetone or chloroform. In the first experiment of this series 50 eyestalks of *Uca* were extracted in acetone. The acetone-soluble fraction which contains a white pigment-concentrating substance, but none of the white pigment-dispersing material (Rao, Fingerman and Bartell, 1967) was discarded. The acetone-insoluble material was dried in a desiccator and then extracted in 10 ml ethanol. The alcohol in the ethanol-soluble fraction was allowed to evaporate at room temperature, then the material was eluted in 0.4 ml of distilled water and chromatographed on Bio-Gel. Similarly an ethanol-soluble fraction of the chloroform-insoluble fraction of 50 eyestalks from *Uca* was prepared and chromatographed. The results shown in Figure 5 for the chloroform-treated and acetone-treated material reveal the absence of activity in the samples from the void

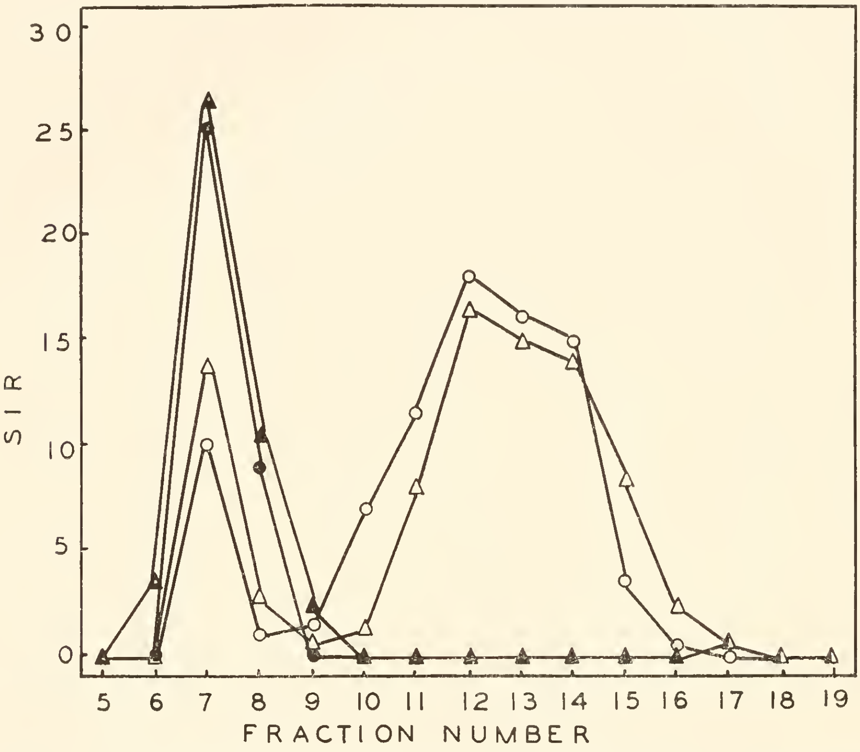

FIGURE 4. The white pigment-dispersing Standard Integrated Responses (SIR) evoked by the fractions of the distilled water eluates of the ethanol-soluble fraction extracted without excessive stirring from the eyestalks of *Uca* (dots) and *Palaemonetes* (solid triangles) and of the ethanol-soluble fractions extracted with excessive stirring from the eyestalks of *Uca* (circles) and *Palaemonetes* (empty triangles) after filtration through a column of Bio-Gel P-6. Other details same as Figure 3.

volume, but the activity appeared later and peaked in fraction 12. Thus, by treatment with chloroform or acetone it was possible to remove the white pigment-dispersing hormone from the heavy component in the ethanol-soluble material.

The aim of the next experiment was to determine the effect of boiling on the white pigment-dispersing substances in an extract prepared directly in water and in the ethanol-soluble fraction of the eyestalks of *Uca* and of *Palaemonetes* as measured by changes in the responses to these substances. Distilled water extracts were made from 50 eyestalks of *Palaemonetes* and 50 eyestalks of *Uca*. The extracts were filtered individually through the column of Bio-Gel P-6 and fraction 12, which produced the most white pigment-dispersing activity of the material retarded by the gel (Figs. 3, 4, 5), was divided into two equal portions. One portion of the extract was placed in a boiling water bath for five minutes while the other portion was left at room temperature. The boiled extract was cooled, made up to the original volume, and then the boiled and unboiled extracts were each assayed for white pigment-dispersing activity on 10 intact crabs adapted to

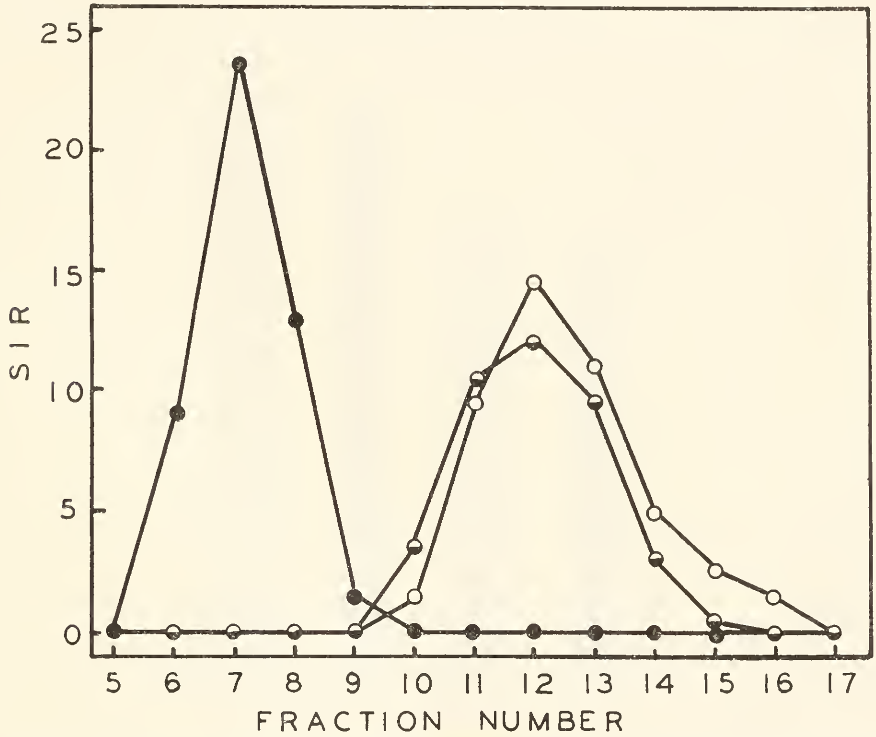

FIGURE 5. The white pigment-dispersing Standard Integrated Responses (SIR) evoked by the various fractions of the distilled water eluates of the ethanol-soluble fraction prepared from fresh eyestalks of *Uca* (dots) and of the distilled water eluates of the ethanol-soluble fraction prepared from the eyestalks of *Uca* that were pre-extracted with acetone (circles) or chloroform (half-filled circles) after filtration through a column of Bio-Gel P-6. Other details same as Figure 3.

a black background. Ethanol-soluble fractions of 50 eyestalks of *Uca* and 50 eyestalks of *Palaemonetes* were then prepared. During the extraction vigorous stirring was employed so as to liberate the white pigment-dispersing substance from the heavy component. After filtering these extracts through Bio-Gel P-6 fraction 12 was boiled as in the previous experiment and assayed for white pigment-dispersing activity. Each experiment was repeated once and the averaged results are shown in Figure 6. The white pigment-dispersing substance in the ethanol-soluble fraction of the eyestalks from *Uca* as well as *Palaemonetes* is thermolabile, whereas the white pigment-dispersing substance in an extract of these eyestalks prepared directly in water is thermostable. The most logical explanation of these results is that we are dealing with at least two different white pigment-dispersing subsances; one, thermostable, is extractable in distilled water, the other, thermolabile, is extractable in ethanol.

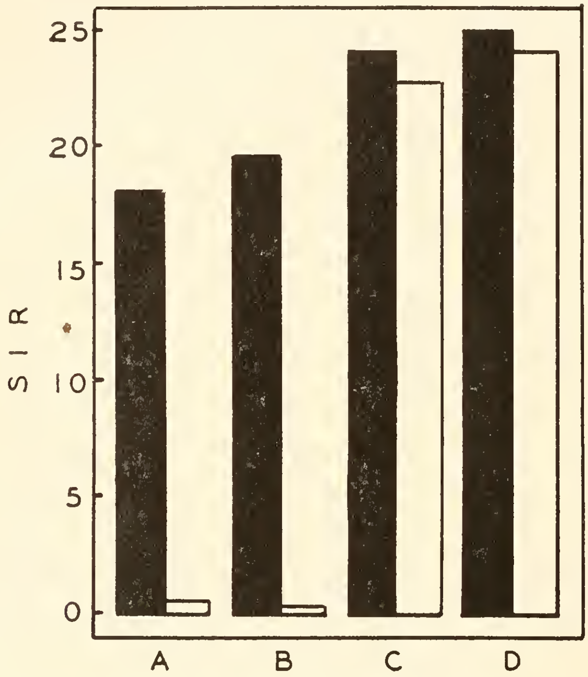

FIGURE 6. The white pigment-dispersing Standard Integrated Repsonses (SIR) evoked by the unboiled (solid bars) and boiled (empty bars) fractions retarded by Bio-Gel P-6. Distilled water eluates of the ethanol-soluble fraction of the eyestalks from *Palaemonetes* (A) and *Uca* (B); distilled water extracts of the eyestalks from *Palaemonetes* (C) and *Uca* (D).

Previous investigation (Rao, Fingerman and Bartell, 1967) revealed that the white pigment-dispersing substance antagonizes the action of the white pigment-concentrating substance. If a white pigment-concentrating substance is present in the ethanol-soluble fraction of eyestalks from *Uca* in spite of the fact that its presence is not revealed following injection of such extracts into eyestalkless crabs (Fig. 1), and if it is thermostable, its presence should become apparent by selectively inactivating the antagonistic thermolabile white pigment-dispersing substance by boiling. The following experiment was conducted to test this conclusion. One hundred twenty eyestalks from *Uca* were extracted in 24 ml ethanol and after centrifugation 22 ml of the supernatant were divided equally among 11 evaporating dishes. The alcohol was allowed to evaporate at room temperature. Then one dish was placed in a desiccator while the others were placed in an oven at 95° C. The dishes were removed one at a time, 2, 4, 6, 8, 12, 14, 16, 18, 24, and 36 hours after they had been placed in the oven. After removal from the oven the sample was placed in a desiccator so that all the samples could be assayed on the same day. Each sample was dissolved in 0.5 ml saline and tested on five eyestalkless *Uca* and five intact *Uca* adapted to a black background. The experiment was repeated once. An aqueous extract was then prepared directly from 120 eyestalks of *Uca* also. This extract was likewise divided into 11 portions of 2 ml each, but they were lyophilized before being treated as in the protocol of the above experiment. The averaged results for these experiments are shown in Figure 7. With increase in the length of exposure to heat the white pigment-dispersing activity

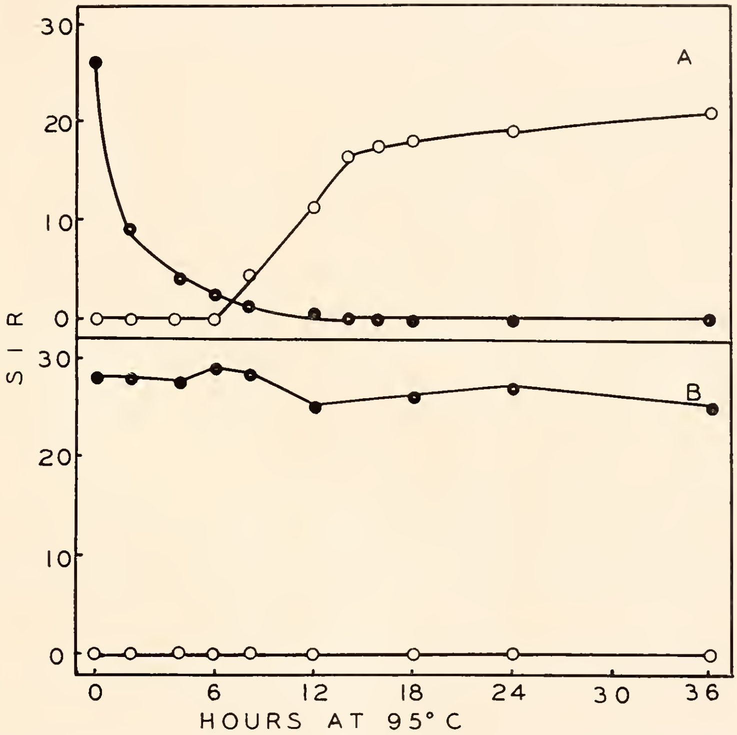

FIGURE 7. The effect of drying the ethanol-soluble fraction of fresh eyestalks of *Uca* (A) and the lyophilized water-soluble fraction of the eyestalks of *Uca* (B) for varying lengths of time in an oven. Dots, white pigment-dispersing Standard Integrated Responses (SIR) ; circles, white pigment-concentrating Standard Integrated Responses. The concentration of each extract was one eyestalk per dose of 0.05 ml.

decreased and completely disappeared in the samples heated for 12 hours and longer. However, concomitantly the white pigment-concentrating activity began to appear and gradually increased with decrease of the white pigment-dispersing activity. In contrast, the material in the extract prepared directly in water always evoked only dispersion of the white pigment. Even if the white pigment-concentrating substance is present in the material extracted directly in water, it is not possible to demonstrate its activity by this method because the antagonistic white pigment-dispersing substance in this fraction is thermostable.

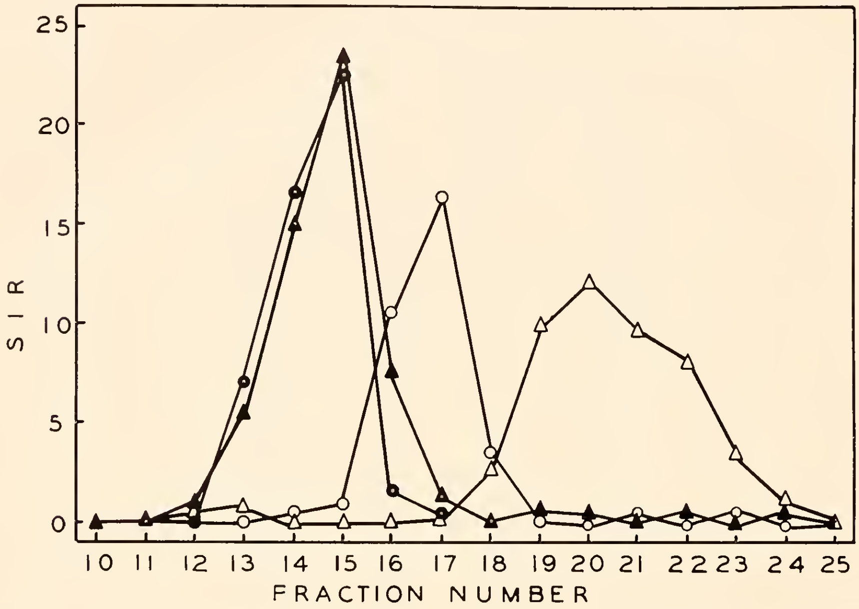

FIGURE 8. The white pigment-dispersing (dots and solid triangles) and white pigment-concentrating (circles and empty triangles) Standard Integrated Responses (SIR) evoked by the fractions of the ethanol-soluble material extracted from the eyestalks of *Uca* (dots and empty triangles) separated by filtration through a 28 × 1.5 cm column of Sephadex LH-20. Flow rate: 30 ml per hour. Sample size: 2 ml.

From the above experiment it is clear that the ethanol-soluble fraction of the eyestalks from *Uca* has both the white pigment-dispersing and -concentrating substances. The ethanol-soluble fraction of the eyestalks from *Palaemonetes* also has both substances (Fig. 2). However, the white pigment-concentrating substance did not appear in any of the fractions obtained after chromatographing the ethanol-soluble fractions of eyestalks from *Uca* and *Palaemonetes* on the Bio-Gel. Consequently in an effort to separate the white pigment-dispersing and -concentrating substances and obtain an estimate of their relative molecular weight it was decided to try another gel. Ethanol extracts were consequently chromatographed on Sephadex LH-20 with ethanol as the solvent. Fifty eyestalks from *Uca* were extracted in 10 ml ethanol and the ethanol-soluble fraction was allowed to evaporate at room temperature. The material was redissolved in 0.4 ml ethanol and then chromatographed on a column of LH-20 in ethanol. The red pigment in the extract peaked in fraction 11 which represents the end of the void volume, and this sample had no effect on the white chromatophores of *Uca* (Fig. 8). The white pigment-dispersing activity peaked at fraction 15, whereas the white pigment-concentrating activity which was then apparent peaked at fraction 17; the substances had been separated. In the next experiment the ethanol-soluble fracion prepared from 50 eyestalks of *Palaemonetes* was filtered through LH-20. The peak of white pigment-dispersing activity coincided with that of the ethanol-soluble fraction of eyestalks from *Uca*. However, the white pigment-concentrating activity in the

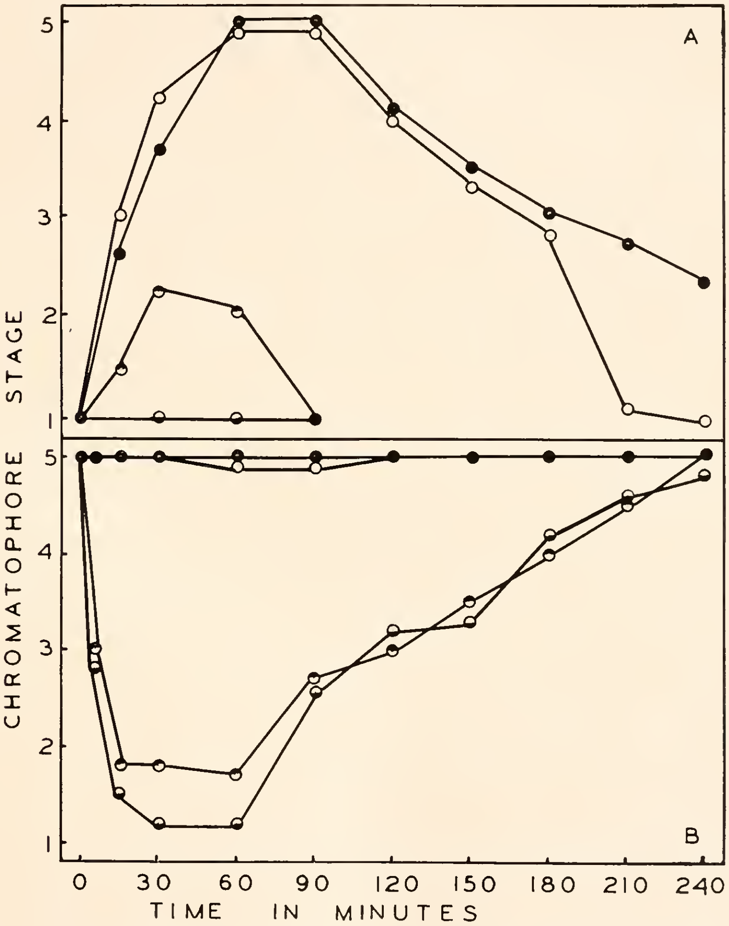

FIGURE 9. The white pigment-dispersing (A) and white pigment-concentrating (B) responses evoked by saline eluates of the untreated ethanol-soluble fraction (dots), isopropyl ether-treated ethanol-soluble fraction (circles with bottom half filled), untreated lyophilized fraction extracted directly in distilled water (circles), and the isopropyl ether-treated lyophilized fraction extracted directly in distilled water (circles with top half filled) from fresh eyestalks of *Uca pugilator*. The concentration of each extract tested was 1 eyestalk per dose of 0.05 ml.

extracts of eyestalks from *Palaemonetes* peaked in fraction 21, much later than did the white pigment-concentrating substance from *Uca* (Fig. 8).

So far no evidence has been obtained as to whether or not a white pigment-concentrating substance is present in extracts of eyestalks from *Uca* prepared in water. Rao, Bartell and Fingerman (1968) showed that the melanin-dispersing substances in the extract prepared directly in water and in the ethanol-soluble fraction of eyestalks from *Uca* were inactivated by isopropyl ether treatment. The following experiment was conducted to determine (a) whether the white pigment-dispersing substances in these extracts can also be inactivated in a similar way and (b) whether the extract prepared directly in water contains both the white pigment-dispersing and -concentrating substances. An extract was prepared directly in 15 ml distilled water from 40 eyestalks of *Uca* and divided into two equal portions which were subsequently lyophilized. One sample which served as the control was stored on dry ice. To the other sample was added 10 ml isopropyl ether and the container was then covered. After an overnight exposure to isopropyl ether the ether was allowed to evaporate at room temperature and the material was dissolved in 1.0 ml saline and injected into 10 eyestalkless crabs and 10 intact crabs adapted to a black background. The control sample was likewise assayed. The ethanol-soluble fraction was then prepared from 40 eyestalks and divided into two equal portions. After the alcohol had evaporated one sample was placed in a desiccator, while the other was treated with isopropyl ether as described above. These treated and untreated samples were then assayed for action on the white chromatophores. The results (Fig. 9) reveal that the white pigment-dispersing substances in the ethanol-soluble fraction of the eyestalks from *Uca* and in the extract prepared directly in water are inactivated by isopropyl ether treatment just as are the melanin-dispersing substances. In the absence of the antagonistic white pigment-dispersing substance, the white pigment-concentrating substance was demonstrable. Therefore, it is clear that the white pigment-dispersing and -concentrating substances are both present in the aqueous and alcoholic extracts of these eyestalks.

Discussion

A previous comparative study (Fingerman and Couch, 1967) of chromatophorotropins from *Uca* and *Palaemonetes* revealed that extracts of eyestalks from *Palaemonetes* evoke pigment migration in the melanophores and erythrophores of *Uca*. The present study revealed that extracts of eyestalks from *Palaemonetes* can evoke dispersion and concentration of the pigment in the leucophores of *Uca* also. Of significance is the question whether pigment dispersion in each type of chromatophore is regulated by a separate substance or whether a single substance evokes pigment dispersion in the melanophores, erythrophores, and leucophores. Brown and Fingerman (1951) have shown that the melanin-dispersing and red pigment-dispersing substances in the supraesophageal ganglia of *Uca* are different. Fingerman and Couch (1967) arrived at the same conclusion for the red pigment-dispersing and melanin-dispersing substances in the eyestalk of *Uca*. Rao, Fingerman and Bartell (1967) found that an extract of the circumesophageal connectives from *Uca* evoked melanin dispersion but not white pigment dispersion in *Uca*, and concluded that it is highly unlikely that both actions are due to a single substance. The latter investigators also found that the white pigment-concentrating substance antagonizes the action of white pigment-dispersing substance but not the melanin-dispersing substance. Further support for the conclusion that dispersion of white pigment and dispersion of melanin are due to different substances comes from the present study. At concentrations of 0.5 eyestalk per dose and above the melanin-dispersing activity of the ethanol-soluble fraction of the eyestalks from *Uca* is much higher than that of an aqueous extract prepared directly from the eyestalks of *Uca* (Rao, Bartell and Fingerman, 1967). In contrast, at all the concentrations tested the white pigment-dispersing activity of the ethanol-soluble fraction of the eyestalks was nearly identical to that of the aqueous extract of the eyestalks (Figs. 1, 2). This observation is also quite different from the situation reported for the crab, *Ocypode macrocera,* where with a concentration of one eyestalk per dose the white pigment-dispersing activity of the ethanol-soluble fraction was 25 times more than that evoked by an extract of the optic ganglia prepared direcly in saline (Rao, 1967). *Uca* and *Ocypode* are members of the same family, the Ocypodidae. Attempts are now underway in this laboratory to separate the melanin-dispersing and white pigment-dispersing substances of *Uca pugilator.*

Injection of an extract of eyestalks from *Uca* prepared directly in saline results in dispersion but not concentration of the pigment in the leucophores of *Uca.* However, acetone fractionation revealed that the white pigment-dispersing and -concentrating substances are both present in the eyestalk (Rao, Fingerman and Bartell, 1967). Two alternative explanations were given. (1) A white pigment-concentrating substance is present in the aqueous extract but its expression is inhibited by the presence of its antagonist, the white pigment-dispersing substance or (2) a white pigment-concentrating substance exists in the nervous tissues in an inactive form and acetone extraction renders it active and soluble in water. The present study lends support to the former view. At all concentrations of the extracts prepared directly in water and the ethanol-soluble fractions of the eyestalks from *Uca* and the extracts of eyestalks from *Palaemonetes* prepared directly in water the white pigment-dispersing substance dominated the white pigment-concentrating substance (Figs. 1, 2). However, at low concentrations of the ethanol-soluble material from the eyestalks of *Palaemonetes* white pigment-concentration was evident, and as the concentration increased the white pigment-dispersing substance dominated more and more the white pigment-concentrating substance (Fig. 2).

The white pigment-concentrating substance in the eyestalk extracts could not be recovered after chromatography on Bio-Gel P-6. This substance was probably adsorbed to the gel matrix. However, chromatography of ethanol extracts of the eyestalks on Sephadex LH-20 yielded good separation of the white pigment-dispersing and -concentrating substances (Fig. 8). The white pigment-concentrating substance in the eyestalks of *Palaemonetes* is a smaller molecule than the white pigment-concentrating substance in the eyestalks of *Uca.* The gel filtration studies indicate that the low molecular weight substances having white pigment-dispersing activity from *Uca* and *Palaemonetes* may not differ in their molecular weights. The observation (Fig. 6) that the white pigment-dispersing substance in the ethanol-soluble fraction is thermolabile while that in the material extracted directly in water is thermostable leads to the conclusion that we are dealing with at least two different substances.

The finding that when distilled water eluates of the ethanol-soluble fraction are filtered through Bio-Gel P-6 (Fig. 4) most of the white pigment-dispersing activity is associated with the material in the void volume is in agreement with previous work on melanin-dispersing substances (Bartell, Rao and Fingerman, 1967). These investigators obtained evidence in favor of the theory that the high molecular weight material in the void volume consists of a complex of an active peptide and a large lipoidal substance. Rao, Bartell and Fingerman (1968) have suggested that this lipid-containing material may be the carrier substance whose presence and nature has been revealed by cytochemical studies of neurosecretory cells in other organisms. Bern and Hagadorn (1965) have reviewed the available information on the chemical nature of the carrier substance. When ethanol extracts were chromatographed on LH-20 (Fig. 8) no activity was found in the void volume, showing that the material while in ethanol is a small molecule.

Although certain substances in the eyestalks of *Palaemonetes* evoke pigment migration in the leucophores of *Uca*, we are unable to determine whether they act on the leucophores of *Palaemonetes* itself. The responses seen in this laboratory of the leucophores in *Palaemonetes* to eyestalk extracts have proven to be extremely erratic and as a consequence no definitive conclusions could be drawn from the data. However, after we are able to develop a suitable technique for investigating the responses of the white chromatophores in *Palaemonetes* further comparative study of the control of the leucophores in these two crustaceans would be worthwhile.

SUMMARY

1. The relationships between the response of the leucophores in *Uca pugilator* and the concentration of extracts of eyestalks from *Uca pugilator* and *Palaemonetes vulgaris* were determined. For the first time evidence is provided to show that substances capable of evoking pigment dispersion and pigment concentration in the leucophores of *Uca* are present in the eyestalks of *Palaemonetes*. Contrary to the situation recorded previously for melanin-dispersing activity, extracts prepared directly in saline and the ethanol-soluble fractions of the eyestalks from *Uca* evoked nearly identical white pigment-dispersing activity at all dilutions tested.

2. White pigment-dispersing and -concentrating substances can both be demonstrated to be present in the ethanol-soluble fraction of the eyestalks of *Uca* as well as in the material directly extractable in water in spite of the fact that when these extracts are assayed immediately after preparation they evoke only white pigment dispersion because the white pigment-dispersing substance antagonizes the action of the white pigment-concentrating substance.

3. The eyestalks of *Uca* as well as *Palaemonetes* contain at least two white pigment-dispersing substances each. The substance in the ethanol-soluble fraction is thermolabile whereas that in the extract prepared by extracting the eyestalks directly in saline is thermostable. However, both are inactivated by prolonged exposure to isopropyl ether.

4. Gel-filtration studies revealed that the white pigment-concentrating substance in the ethanol-soluble fraction of the eyestalks of *Palaemonetes* is a smaller molecule than the white pigment-concentrating substance in the ethanol-soluble fraction of the eyestalks of *Uca*, but there is no reason to consider the correspond-

ing white pigment-dispersing substances in the extracts prepared directly in saline, and in the ethanol-soluble fractions from both species as different from each other.

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STUDIES ON OÖGENESIS IN THE POLYCHAETE ANNELID *NEREIS GRUBEI* KINBERG. I. SOME ASPECTS OF RNA SYNTHESIS

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Oögenesis in the marine polychaete *Nereis* appears to include at least two distinct growth phases. Oöcyte growth is slow for much of the period available for oögenesis, and more rapid during the period of somatic metamorphosis which precedes spawning (Clark and Ruston, 1963). Both somatic metamorphosis and rapid oöcyte growth can be initiated by decapitation, which is thought to deprive the animal of a prostomial source of an inhibitory hormone (Durchon, 1952; Hauenschild, 1956). Inasmuch as the suggestion has been made that an alteration in RNA metabolism is associated with the increased growth rate of the oöcytes (Durchon and Boilly, 1964), we have examined the types of RNA normally formed during the rapid growth period, which in *Nereis grubei* commences when the oöcytes are 90–100 μ in diameter. Oöcyte morphology changes considerably through this period (Spek, 1930); in *N. grubei* the oöcyte is blue-green and opaque for most of the period of rapid growth. When it reaches 170–180 μ in diameter, the color changes to a brilliant yellow-green. Still later, the lipid yolk coalesces into large translucent droplets around the nucleus, while granules of jelly precursor material condense at the cortex of the oöcyte, which is about 200 μ in diameter at maturity. Mature oöcytes thus contain a yellow-green and strikingly layered cytoplasm, and are distinct in appearance from the opaque, homogeneous, blue-green 160 μ oöcytes. RNA synthesis during the rapid growth period has been examined in cells in each of these morphological stages.

MATERIALS AND METHODS

Animals

Female specimens of *N. grubei* of appropriate age were collected at several points in central California (Moss Beach, San Mateo County; Pacific Grove, Monterey County). They were maintained in the laboratory at 11–15° C in sea water containing 100 μg/ml streptomycin base and 100 units/ml penicillin.

Incubation of oöcytes with tritiated uridine

Animals anesthetized in 7.3% $MgCl_2$ were passed through a series of five changes of sterile sea water, with vigorous agitation between changes. The animal was then split open; the oöcytes were removed in as sterile a manner as possible, placed upon sterile Nitex nylon bolting cloth (44 μ or 64 μ mesh) and rinsed repeatedly with sterile sea water until phase microscopic examination indicated the absence of all coelomic cells. The oöcytes were then placed in 5 ml sterile sea water containing 35 μc of H^3-uridine (New England Nuclear. Specific activities are given in the figure legends).

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Those species of *Nereis* which breed in the heteronereid state appear to swarm soon after the oöcytes have reached the mature condition. Females deprived of males during this period will swim actively for a period of hours, then sink to the bottom. Soon thereafter, the oöcytes and the whole coelomic mass become gelatinous and the oöcytes begin to degenerate. The "fresh mature" oöcytes were taken from an animal actively swimming at the time of oöcyte removal. The "old mature" oöcytes were from an animal which had been swimming the previous day. These cells maintained excellent morphological integrity, but could be expected to disintegrate within 24–48 hours.

Upon completion of the incubation period, an aliquot of cells was withdrawn and fixed for one hour in acetic acid-ethanol (1:3) for autoradiography. The balance was frozen in a dry-ice-acetone bath for subsequent RNA extraction.

Autoradiography

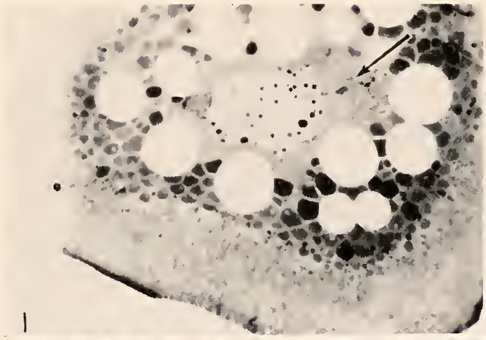
After fixation the oöcytes were stained lightly with eosin to enhance visibility, imbedded in paraffin and sectioned at 5 μ. After paraffin removal, the sections were dipped in ice-cold 5% trichloroacetic acid for 15 minutes (not longer), rinsed, and coated with Ilford K5 liquid nuclear emulsion. Exposure was from 10 days to 3 weeks. They were then developed for 6 minutes in Kodak D19 (18° C) and stained in Mayer's hemalum and celestine blue (Doniach and Pelc, 1950). Successful RNase controls required the use of 1 mg/ml RNase (Worthington) for 17 hours (*i.e.* overnight) at 37° C. [Tweedell (1966) also found somewhat extreme conditions to be necessary for this reaction in *Pectinaria* oöcytes.]

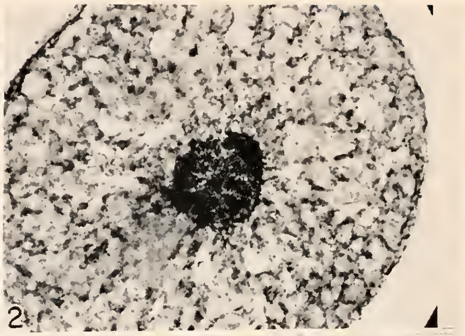
RNA extraction

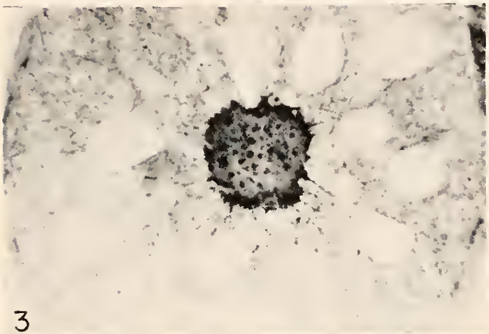
RNA was extracted by the cold-phenol method of Brown and Littna (1964), including DNase digestion (Worthington, 1x crystallized). The concentration of sodium dodecacyl sulfate for the initial phenol extraction was increased to 2%. With two exceptions (the preparations in Figures 8b and 8c), the samples were also digested with 50–100 μg Pronase (Calbiochem; previously autodigested) for an additional 30 minutes at 18–20° C. The samples were then brought to 0.5% SDS and 0.1 *M* NaCl and re-extracted with an equal volume of ice-cold phenol. The aqueous layer was re-extracted with chloroform at least twice more, and the RNA again precipitated with the addition of two volumes of absolute ethanol.

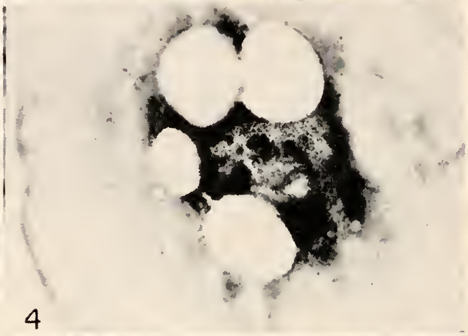
Analysis of extracted RNA

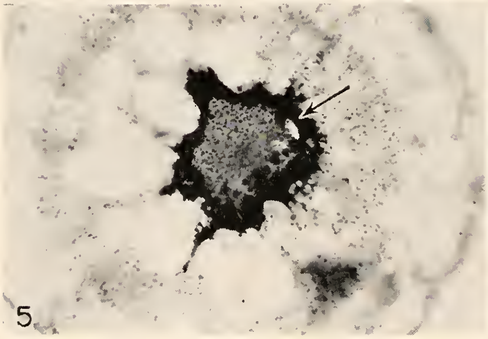
For sucrose gradient analysis, aliquots (1 ml or less) of the RNA solutions in .01 *M* sodium acetate, pH 5 were layered on top of 25 ml linear 5–20% sucrose gradients, made up in 0.01 *M* sodium acetate, 0.10 *M* NaCl and 10^{-4} *M* EDTA.











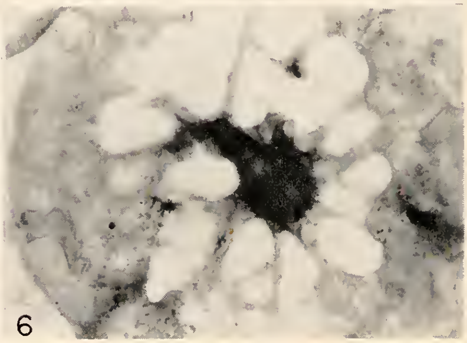


FIGURE 1. One-micron section of a mature oöcyte from *Nereis grubei* imbedded in maraglas and stained with azure II-methylene blue. Note the reticulated area of cytoplasm surrounding the nucleus but clearly distinct from it (arrow).

FIGURE 2. Autoradiogram of 165 μ oöcyte (G485), blue-green in life, showing nuclear localization of silver grains.

FIGURE 3. Autoradiogram of 190 μ oöcyte (G257), yellow-green in life and showing concentration of lipid yolk but incomplete cytoplasmic stratification. Nuclear localization of silver grains, with local concentrations above nucleoli.

FIGURE 4. Autoradiogram of mature oöcyte (G359) incubated 4 hours with H^3-uridine. Nuclear concentration of label, with relatively little over basophilic perinuclear cytoplasm.

FIGURE 5. Autoradiogram of older mature oöcyte (G548) with both nuclear and cytoplasmic label; the cytoplasmic label is concentrated over the basophilic perinuclear cytoplasm.

FIGURE 6. Autoradiogram of oöcyte from same sample as Figure 5, treated 17 hours with RNAase. Slight residual nuclear label, disappearance of nucleoli, perinuclear cytoplasmic basophilia and most silver grains.

Aliquots for RNase digestion were adjusted to pH 8 with 0.1 *M* Tris-HCl and incubated 10 minutes or longer at 18–20° C with 10–20 μg of boiled RNase (Worthington). The extracted RNA showed none of the resistance to the enzyme shown by that in the autoradiograms. The gradients were centrifuged 9–10 hours at 2° C and 25,000 RPM in the SB-110 rotor of an International B-60 ultracentrifuge. Fractions were diluted to 3 ml with 0.01 *M* sodium acetate, pH 5, for determination of the optical density at 260 mμ in a Beckman DU spectrophotometer, precipitated in the cold with 0.1 mg bovine albumen carrier and 0.3 ml 50% trichloroacetic acid, collected on glass fiber filters (Reeve-Angel) and counted in a Nuclear-Chicago liquid scintillation spectrometer with 4 ml toluene scintillator (4 g PPO and 0.2 g POPOP per liter of toluene).

DNA base composition

Two gamete-bearing male *Nereis* were homogenized in 5 ml SET (0.1 *M* NaCl, 0.1 *M* EDTA, 0.01 *M* Tris-HCl, pH 8) and warmed immediately to 60° C. Twelve mg Pronase (Calbiochem; previously autodigested) and sodium dodecyl sulfate to 0.5% were added, and incubated for 2½ hours. The solution was then cooled to room temperature and extracted by gentle rocking with an equal volume of water-saturated phenol. Following centrifugation at 12,000 g the aqueous layer was removed, re-extracted with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged as before. Two volumes of absolute ethanol were added to the aqueous extract; the precipitate was collected and redissolved in 4 ml SET diluted 1:1 with water. The solution was then incubated with 200 μg RNase (Worthington; previously boiled 10 minutes) for 2½ hours at 37° C. Following addition of 500 μg Pronase, the incubation was continued for an additional hour. The DNA was then precipitated with ethanol, redissolved in SET, and extracted twice with phenol and once with chloroform-isoamyl alcohol as before. The resulting ethanol precipitate was redissolved in SET for CsCl density gradient analysis. We are indebted to Dr. Philip Hanawalt for carrying out this analysis. The guanidine-cytidine content of the DNA was calculated from its buoyant density in CsCl by the method of Schildkraut, Marmur and Doty (1962).

RESULTS

Buoyant density of Nereis *DNA*

The buoyant density in CsCl of a sample of *Nereis* DNA extracted as described above was found to be about 1.700 g cc^{-3}, which corresponds to a guanidine-cytidine content of 40–41%. Although this information could not be put to the use originally intended in this analysis, we feel that it may be helpful to record this figure for the benefit of future investigations of nucleic acid metabolism in *Nereis*.

Autoradiography

The autoradiograms indicate the presence of labeled uridine in the nucleoli of cells in each size class. Label is also present over non-nucleolar regions of the germinal vesicle in all samples (Figs. 2–5). However, prominent cytoplasmic label

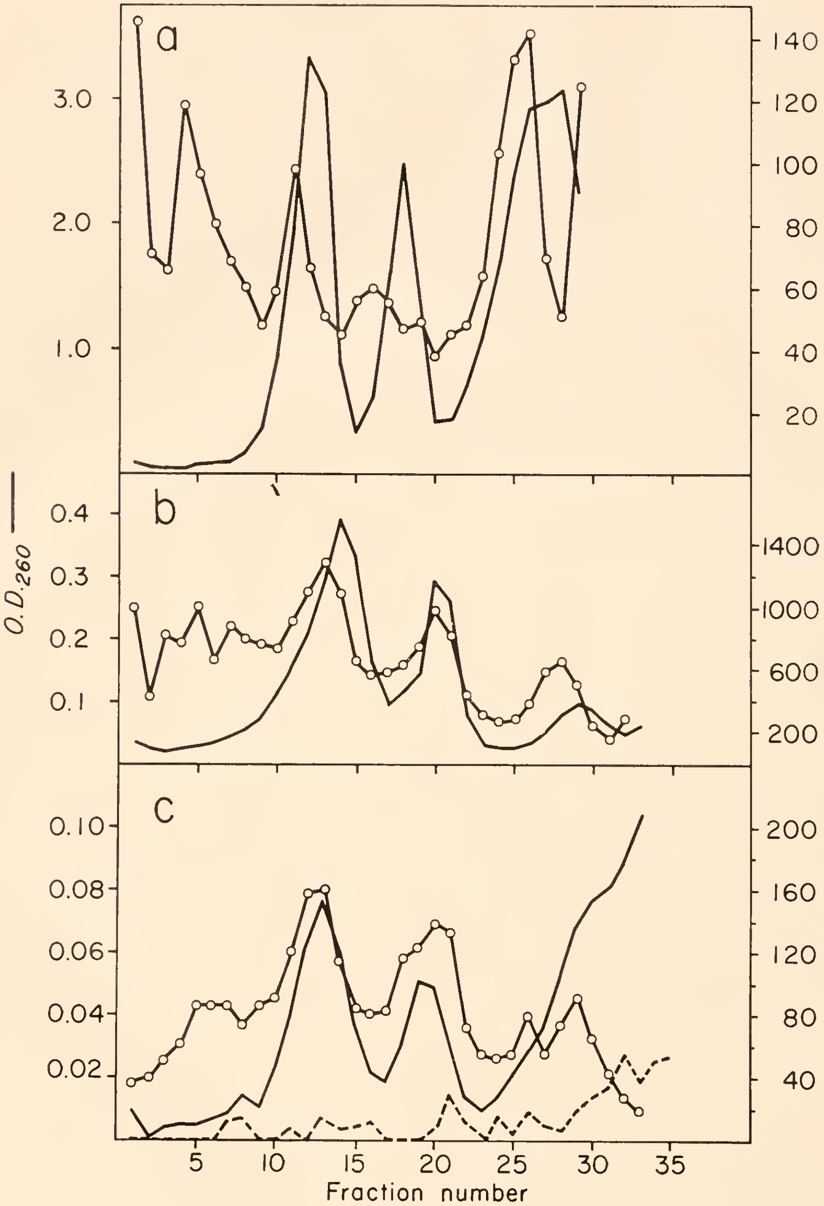


FIGURE 7. Sucrose gradient analysis of RNA from oöcytes of immature *Nereis grubei*. Open circles: radioactivity; solid line: optical density at 260 mμ; broken line: radioactivity after treatment with RNase. Specific activity of H^3-uridine applied in each case is given in parentheses following the incubation time. (a) 100 μ oöcytes, 4 hours incubation (24.5 C/m*M*). *Urechis caupo* carrier RNA added. (b) 165 μ oöcytes, 4 hours incubation (3.6 C/m*M*). No carrier RNA added. (c) 190 μ oöcytes, 4 hours incubation (24.5 C/m*M*). No carrier RNA added.

was found only in the two samples of mature oöcytes incubated for 21 hours with the precursor.

One-micron sections of mature oöcytes imbedded in Maraglas (Fig. 1, arrow) reveal that the basophilic region seen at the nuclear margin in paraffin sections (*e.g.* Fig. 5, arrow) is actually cytoplasmic. This basophilia is present to only a limited extent in 190 μ oöcytes (Fig. 3) and appears to represent a facet of the general layering of cytoplasmic elements which occurs during the final stage of oöcyte development. Cytoplasmic labeling in mature oöcytes incubated for 21 hours with H^3-uridine is concentrated in this region of the cytoplasm (Fig. 5) although the region is only slightly labeled in mature oöcytes incubated for four hours (Fig. 4). Both the incorporated radioactivity and the basophilia of this region are removed by treatment with RNase (Fig. 6). A preliminary examination of this region with the electron microscope indicates that it does not consist of the stacked annulate lamellae (also basophilic) found in artificially accelerated oöcytes of *Nereis diversicolor* by Durchon and Boilly (1964); (Durchon, 1967, p. 130 and our own observation).

Sedimentation analysis of extracted RNA

The sedimentation patterns of RNA extracted from *Nereis* oöcytes are presented in Figures 7 and 8. The RNA synthesized by immature and mature oöcytes during a 4-hour incubation with H^3-uridine is distinctly heterogeneous (Figs. 7a and 8a). However, prominent peaks of radioactivity are associated with the 28s and 18s ribosomal RNAs (sedimentation values have not been determined for *Nereis* RNAs, but are given with reference to RNA from other eukaryotic cells) seen by optical density in the preparations from 165 μ and 190 μ oöcytes (Figs. 7b and 7c) and from mature oöcytes labeled for 21 hours (Figs. 8b and 8c). Furthermore the radioactivity profile from the 100 μ oöcytes is consistent with the presence of 45s and 30s ribosomal RNA precursors. Taken together, the sucrose gradient profiles and autoradiograms suggest that ribosomal RNA synthesis occurs at all stages examined.

The low molecular weight RNA synthesized by these oöcytes is not adequately characterized by sucrose gradients; therefore, positive statements about 5s ribosomal (Brown and Littna, 1966) and 4s transfer RNA synthesis are precluded. However, it does appear that mature oöcytes accumulate relatively little radioactivity into low molecular weight RNA when compared with younger oöcytes. This observation could reflect a drastic reduction or cessation of transfer RNA synthesis towards the end of oögenesis.

No differences were found which could be clearly associated with the progressively altered morphology of the oöcytes.

In evaluating these results, the fact that the oöcytes were incubated in sea water rather than coelomic fluid should be kept in mind. We have observed that eleocytes adhering to growing coelomic oöcytes readily separate from them upon transfer to sea water, indicating some change at the oöcyte surface. Furthermore, Gonse (1957a, b) has reported differences in respiratory levels in oöcytes from the sipunculid *Phascolosoma vulgare* which he attributes to an effect of sea water upon cell permeability to sugars.

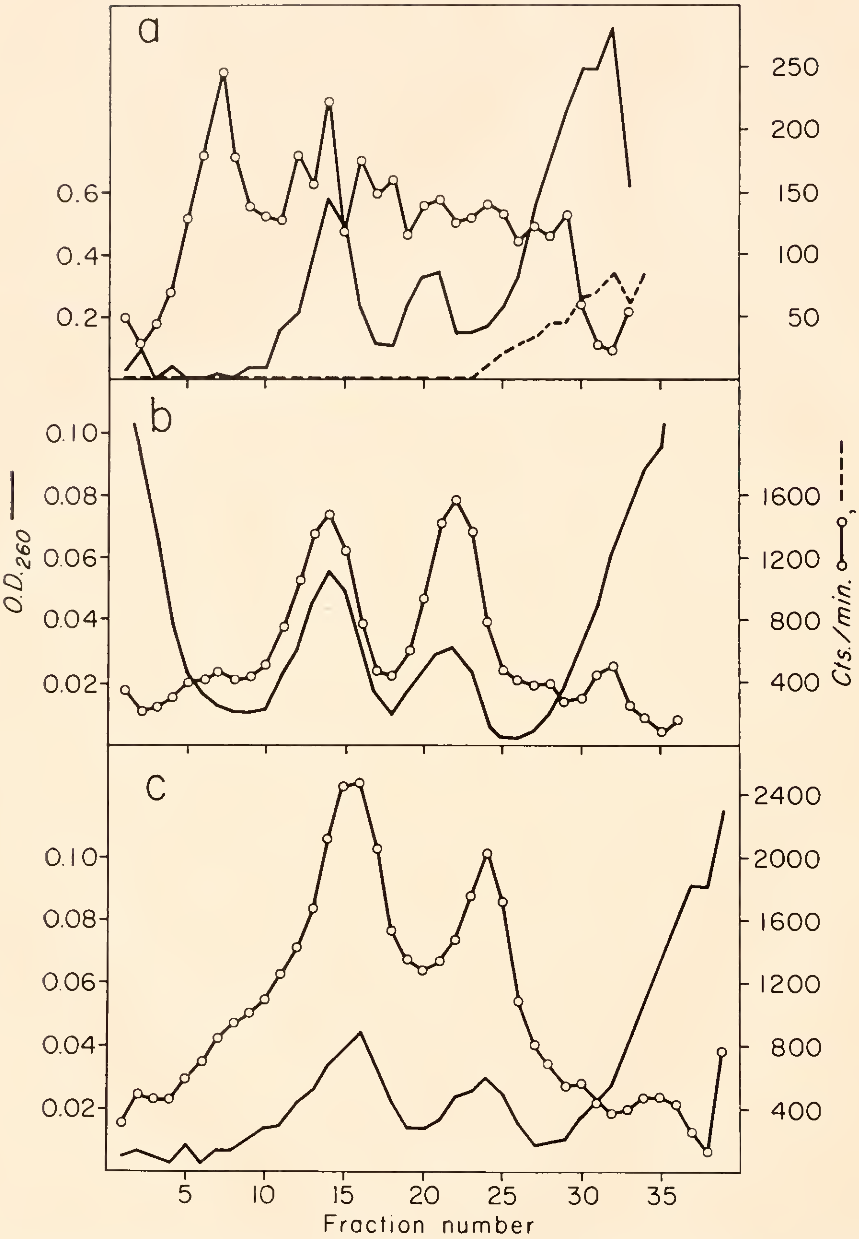


FIGURE 8. Sucrose gradient analysis of RNA from mature oöcytes. Legend as for Fig. 7. (a) Mature oöcytes 4 hours incubation (24.5 C/m*M*). No carrier RNA added. (b) "Fresh" mature oöcytes, 21 hours incubation (8 C/m*M*). No carrier RNA added. (c) "Older" mature oöcytes, 21 hours incubation (8 C/m*M*). No carrier RNA added.

Discussion

These studies clearly demonstrate that mature *Nereis* oöcytes, as well as immature oöcytes at the stages examined, are able to synthesize RNA in sea water in the absence of any accessory cells. An active synthesis of RNA has also been observed in the immature oöcytes from a variety of other marine invertebrates. As demonstrated autoradiographically these include sea urchins (Ficq, 1964; Piatigorsky and Tyler, 1967), starfish (Ficq, 1955b), amphibians (Ficq, 1955a), the polychaete worms *Pectinaria* (Tweedell, 1966), *Autolytus* (Allen, 1967) and *Ophryotrocha* (Ruthmann, 1964), and the echiuroid worm *Urechis* (Gould, unpublished data). Sucrose gradient analysis of the RNA synthesized by immature sea urchin (Gross, Malkin and Hubbard, 1965; Piatigorsky and Tyler, 1967) and *Urechis* (Gould, unpublished data) oöcytes has indicated considerable ribosomal RNA synthesis.

Reports of RNA synthesis by mature oöcytes, however, are fewer. Ribosomal RNA, transfer RNA, and RNA with a more DNA-like base composition are synthesized by mature *Urechis* oöcytes (Gould, unpublished data). Small amounts of heterogeneously sedimenting radioactive RNA have been detected in mature sea urchin oöcytes (Siekevitz, Maggio and Catalano, 1966), but studies with mature sea urchin oöcytes have generally been frustrated by the poor penetration of RNA precursors into these eggs (Piatigorsky, Ozaki and Tyler, 1967). The finding that mature *Nereis* oöcytes incorporate considerable amounts of precursor into RNA further discredits the idea that all mature oöcytes are in a state of metabolic inhibition (see, for example, Monroy, 1965, p. 77).

It should be noted, however, that our results contrast somewhat with the autoradiographic observations of Dhainaut (1965) on the oöcytes of the non-metamorphosing *Nereis diversicolor*. Mature oöcytes (200 μ) of this species were found to contain little cytoplasmic label and relatively little nuclear label after 12 hours in the presence of injected H^3-uridine. This author concludes that RNA synthesis virtually ceases in the mature oöcytes of this species, although we have found notable incorporation in three runs with mature oöcytes from *N. grubei*, which metamorphoses to spawn. It would be interesting to know whether this difference is related to the different modes of reproduction in the two species, or to differences of technique.

Previous investigators have found it difficult to study RNA metabolism in immature oöcytes of a given stage in marine invertebrates. Thus Piatigorsky *et al.* (1967) reported results obtained with mixtures of mature oöcytes and immature oöcytes of variable age, obtained from the sea urchin *Lytechinus pictus*. These authors have also examined the RNA accumulated in mature oöcytes gathered from animals injected with the precursor weeks or months previously (Piatigorsky and Tyler, 1967). However, the study of synthetic processes during echinoid oögenesis is hampered by the nature of the sea urchin ovary, in which oöcytes develop asynchronously while imbedded in a matrix of several other cell types (Holland and Giese, 1965). Oöcytes in a given stage of development cannot be conveniently isolated for chemical analysis. The ease with which relatively homogeneous populations of oöcytes in a given stage of oögenesis may be obtained makes *Nereis* an excellent organism in which to investigate the stage specificity of the synthesis of ribosomes and other materials (*e.g.* yolk components) which accumulate during oögenesis. The fact that the growth rate of these cells is influenced by an inhibitory hormone also makes them attractive biological objects as a hormone target tissue consisting of a single, isolable cell type.

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SUMMARY

1. RNA synthesis in developing oöcytes of *Nereis grubei* has been studied by autoradiography and by sucrose gradient centrifugation of RNA extracted from oöcytes incubated with tritiated uridine.

2. Oöcyte samples with an average diameter of 100 μ, 165 μ and 190 μ, as well as mature oöcytes (200 μ), all synthesize ribosomal RNA. Labeled uridine was incorporated in the nucleoli of oöcytes from each size class.

3. Oöcytes of all the above stages synthesized heterogeneously sedimenting RNA; mature oöcytes accumulated relatively little label into low molecular weight RNA.

4. No differences in synthetic pattern were found which could be associated with the progressively altered morphology of the oöcytes, but a ribonuclease-sensitive basophilic region, which surrounds the nucleus of mature oöcytes, was found to accumulate label.

5. The cesium chloride buoyant density of DNA isolated from *N. grubei* sperm was found to be about 1.700 g/cc^{-3}, which corresponds to a guanidine-cytidine content of 40–41%.

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MORPHOLOGICAL FEATURES OF FUNCTIONAL SIGNIFICANCE IN THE GILLS OF THE SPINY DOGFISH, *SQUALUS ACANTHIAS*

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A great many studies have now been made concerning the physiology of the gills of the spiny dogfish, *Squalus acanthias*. In addition work has been published under the names *Squalus suckleyi*, *Squalus lebruni* and *Acanthias vulgaris*, all of which are probably synonymous with *Squalus acanthias* (Bigelow and Schroeder, 1948).

Papers have dealt with the flow of water through the branchial chamber (Balabai, 1939; Lenfant and Johansen, 1966), the fall in blood pressure across the gills (Burger and Bradley, 1951), the persistence of the pulse wave into the dorsal aorta (Sheldon, Sheldon and Sheldon, 1962). Study of the exchange of materials between blood and water includes urea (Smith, 1929; Boylan, 1967; Goldstein and Forster, 1962); thiourea (Boylan, 1967); water (Smith, 1931; Boylan, Johnson and Antkowiak, 1962); Na^{22} (Burger and Tosteson, 1966; Horowicz and Burger, 1968); O_2, CO_2, bicarbonate and lactate (Robin, Murdaugh and Millen, 1966; Murdaugh and Robin, 1967); maintenance of osmotic status (Boylan, Kim, Farber and Gerstein, 1965); various organic compounds (Rall, Bachur and Ratner, 1966); and various drugs (Maren, Embry and Broder, 1966). These references suggest the range of studies which have been made although they are by no means a complete listing.

Through these and other publications on the spiny dogfish respiratory system, there runs a vein of vagueness as to the morphology of the gill and the relation of this to the physiological processes involved. For example, Burger and Bradley refer to branchial capillaries, but place quotation marks around the latter word without indicating the nature of the blood channels. Robin and Murdaugh (1967) in their chapter in "Sharks, Skates, and Rays" (page 222) refer to the "gill capillaries." Sheldon, Sheldon and Sheldon (1962) likewise refer to the capillaries of the lamellae. The present paper demonstrates that no capillaries are involved.

Other areas in which the morphology of the gill has special significance include the relatively small fall in blood pressure across the gill, the persistence of the pulse wave into the dorsal aorta, the means whereby large amounts of blood are accommodated, the muscular structure of the arteries, the possible presence of a countercurrent situation and the nature of the epithelium lining some of the water passages.

MATERIALS AND METHODS

The basic method of investigation has been study of serially sectioned gills. Pieces from the approximate center of the gill were cut serially in one of three planes: (1) perpendicular to the surface of the gill and to the filaments (2) perpendicular to the surface of the gill, but parallel with the filaments; (3) parallel to the surface of the gill and hence parallel with the filaments also. With gills of smaller fish complete serial sections were made from pieces of gill extending from the gill arch to the distal tips of the filaments, thus including a sample of the entire length of the filaments.

Explanation of Lettering

BA	basal artery
BC	branchial chamber
CB	cavernous body
CR	cartilaginous ray
CTC	connective tissue core of filament
DA	distal artery
DS	dorso-lateral surface of body
FI	filament (primary lamella)
GS	gill slit
LN	large nerve
LS	lymph space
SE	septum (interbranchial)
SK	skin
SL	secondary lamella
SM	striated muscle
SN	small nerve
VS	ventro-lateral surface of body
WBC	wall of branchial chamber
WC	expanded water channel
WS	water space between filaments or between secondary lamellae

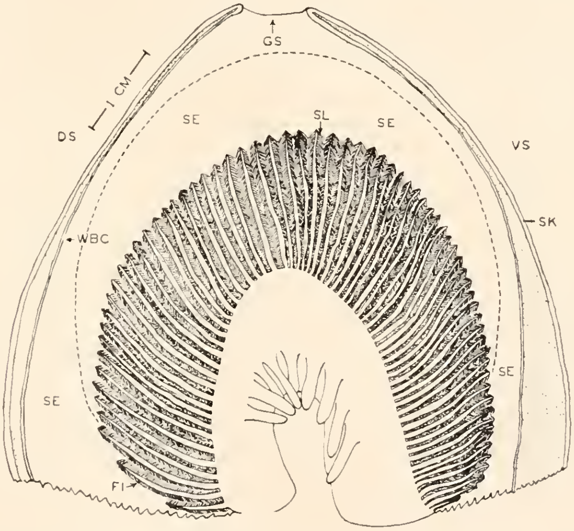

Figure I. Face view of anterior hemibranch of gill number 4 (posterior hemibranch of gill pouch IV, considering the spiracle as pouch I). The broken line indicates the outer edge of the hemibranch on the posterior side of the septum. All microscope drawings made with the aid of projection, finer details being added free-hand during observation of the slide. For explanation of lettering in this and in all following figures see above.

The course of blood through the gill was followed by injecting dilute india ink into the ventricle and allowing 15 to 60 seconds to elapse before clamping the ventral aorta and removing the gill for fixation.

In general alternate slides of serial sections were stained with Delafield's haematoxylin and eosin, and with one or another version of Mallory's connective tissue stain. Some nerve impregnations were made and the material serially sectioned in the three different planes.

The Structure of the Gill

Gross structure

Each group of gill filaments is attached to one of the interbranchial septa separating gill pouches. Thus each gill pouch (except the most posterior) has part of a gill (a hemibranch) on both its anterior and posterior faces. However, a gill consists not of the two hemibranchs in one pouch, but the two which are attached to the opposite sides of the interbranchial septum. Unlike the situation in the teleosts the two hemibranchs of a gill are completely separated from one another, because the septum extends to the surface and is continuous with the skin.

Each hemibranch is composed of a number of ridges or filaments extending from the base of the gill toward its outer edge (Fig. 1). Due to the fact that the septum is not in the transverse plane of the body, but slants posteriorly as it extends outward, and the further fact that both hemibranchs end at approximately the same distance from the gill openings, it follows that the attachment of the hemibranchs is in a somewhat different position on the two sides of the septum (Fig. 1). When a section is made across the gill perpendicular to the filaments, those of the posterior hemibranch are cut closer to their proximal end. Since the filaments are arranged radially and become more separated as they pass distally, a given x-section through the gill will cut more filaments of the posterior than of the anterior hemibranch.

General topography

The general topography of the gill is illustrated in the stereogram (Fig. 2). This shows a portion of one gill, with the septum supporting six filaments on its anterior face and seven and a half on the posterior. The septum, supported by two elliptical cartilaginous rays, contains bundles of striated muscle fibers, two large nerves, and a considerable number of arteries which arise from the trematic branch of the afferent branchial artery. On the anterior face of the septum the arteries are arranged with a high degree of regularity; on the posterior face the presence of the cartilaginous rays interferes with the regular distribution of the arteries. The difference is due to the fact that the rays are not in the middle of the septum but are closer to the posterior surface.

Paralleling the arteries, and connecting with them at intervals, are somewhat rectangular structures which we shall term cavernous bodies (Dröscher, 1882). In one filament of the anterior hemibranch in Figure 2 the connection between the two structures is indicated. An artery and its accompanying cavernous body, extends to the very tip of each filament.

Between adjacent cavernous bodies is a water-filled space, bounded by an epithelium of large cells. This enlarged water passage continues the length of the filaments and is continuous also with water spaces between the secondary lamellae and between adjacent filaments. Typically there is a small nerve, containing eight to ten fibers, below the epithelium in the center of each large water passage.

The free surface of the filament is shaped like half a cylinder. Its outer covering is a thick epithelium, very richly supplied with mucous cells. This cap encloses a core of dense connective tissue within which are typically two cavities which appear to be lymph spaces and occasionally contain small clumps of red blood cells. It also includes a small nerve containing only six to eight fibers and an artery which we shall call the distal artery. The last connects at the base of the gill with the collecting vessel of the efferent branchial artery. Each secondary lamella empties into a distal artery.

Connecting the distal mass of connective tissue with the wall of the cavernous body is a double sheet of connective tissue. In the space between these layers there is an occasional red cell; the space also appears to be a pathway for nerves passing between the basal and distal parts of the filament. The drawing of these structures (Fig. 2) is based on a projection of a cross section of the gill, and is an accurate representation. However, the secondary lamellae are distorted greatly for the sake of clarity. They are attached along the connective tissue sheets in the center of the filament, and extend from the outer part of the cavernous body to the

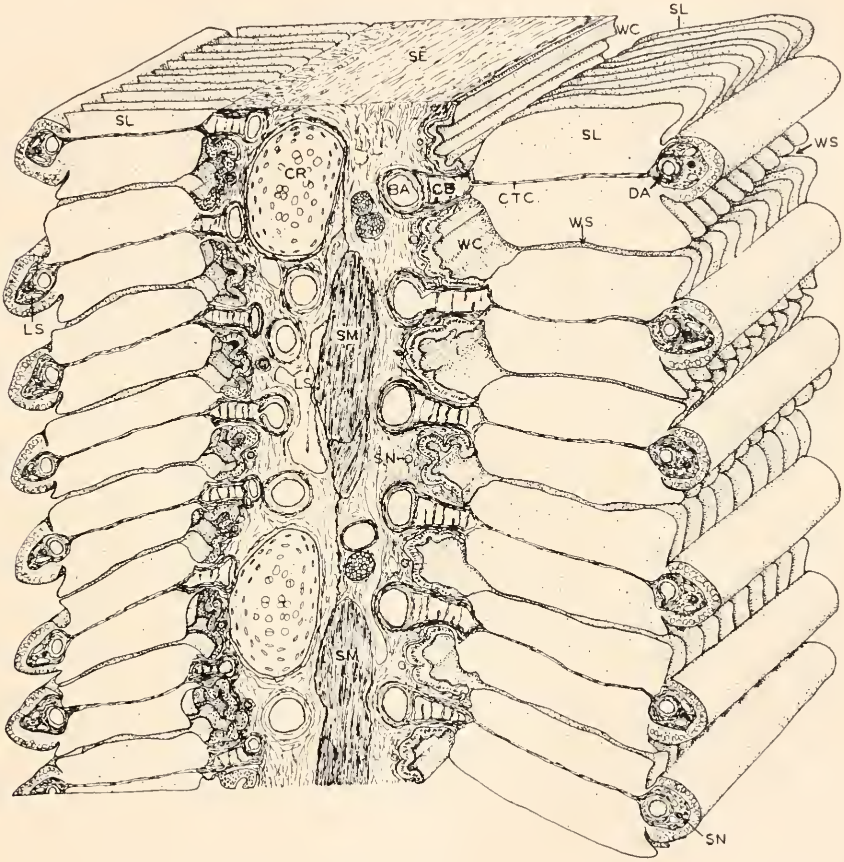

FIGURE 2. Stereogram of a portion of the gill. The front surface is a cross section through the gill perpendicular to the filaments. The anterior hemibranch is on the right, with parts of six filaments; the posterior hemibranch shows parts of eight filaments. The secondary lamellae are greatly simplified in this diagram. In actuality they are set at such an angle to the filaments that they appear in a cross section as they are seen in Figure 3.

distal artery. The distortion consists of representing these as being perpendicular to the septum and to the connective tissue layer. Actually they are set at angles.

The correct relationships appear in Figure 3, which is an accurate drawing of a cross section through one filament. Because of the angle at which the lamellae are attached, parts of seven are visible on each side. Red blood cells are omitted, and the space they occupied is represented by stippling for the sake of emphasis. The dark spots are accurate representations of flecks of ink resulting from a cardiac injection. The connection between the basal artery and the cavernous body is not seen, but there is a pair of connections between the cavernous body and secondary lamellae. The entrance of a secondary lamella into the distal artery is clear. The space between the ends of the secondary lamellae of two adjacent filaments is shown, and the expanded water channels are indicated. The separation of the thick epithelium from the underlying tissue is an artifact.

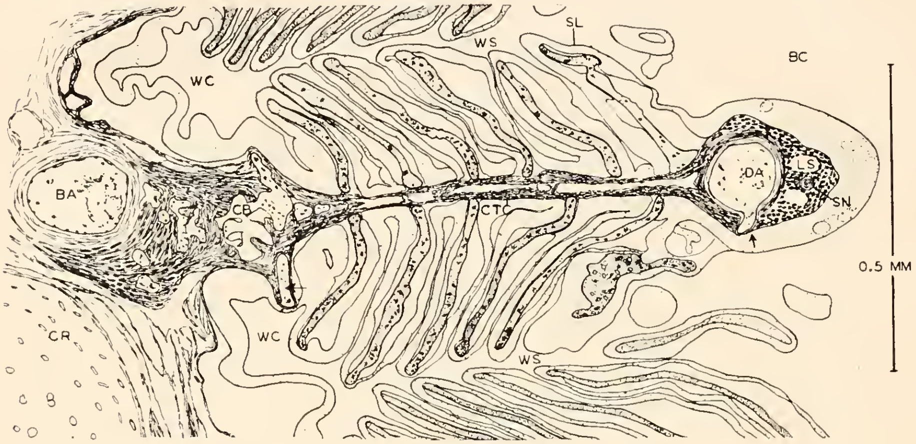

Figure 3. Cross section of filament from a posterior hemibranch. Red blood cells are omitted for clarity; the blood spaces are stippled more heavily than the epithelium, and contain flecks of ink injected into the heart. Arrows indicate connections between the secondary lamella and both the cavernous body and the distal artery.

The cavernous body

The cavity of the cavernous body appears to be divided into separate chambers by complete and incomplete partitions, when seen in sections perpendicular to the filaments (Fig. 4) or in sections parallel to the surface of the hemibranch. However, when examined in a section vertical to the surface but parallel to the filament, it is seen that these are not partitions but columns (Figs. 5 and 6). Each has a core to which large cells are attached, many of which contain pigment granules. While the number of granules is not very impressive in sections, they are present in sufficient number to impart a gray appearance to the entire organ when the gill is washed free of blood by the injection of saline. There is no indication of haemolysis of red cells as described by Acrivo (1935) in *Scyllium canicula.* The nature of the core is not entirely certain. In our preparations there is no reason to believe

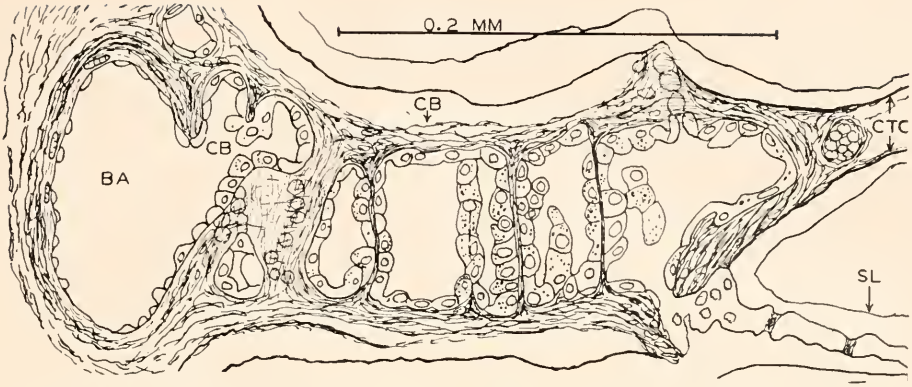

FIGURE 4. Basal artery and cavernous body. From a section perpendicular to the filament. Section is through opening between basal artery and cavernous body; and through the opening from the cavernous body to one secondary lamella. Pigment granules are placed accurately; red cells are omitted. Also omitted are cellular details of the epithelium which covers the secondary lamella and continues as the lining of the expanded water channel.

the core to be other than connective tissue. However, Sheldon, Sheldon and Sheldon (1962) described it as containing smooth muscle fibers.

The thick wall is composed mainly of a rather dense non-elastic connective tissue. This is continuous with the connective tissue surrounding the connecting artery,

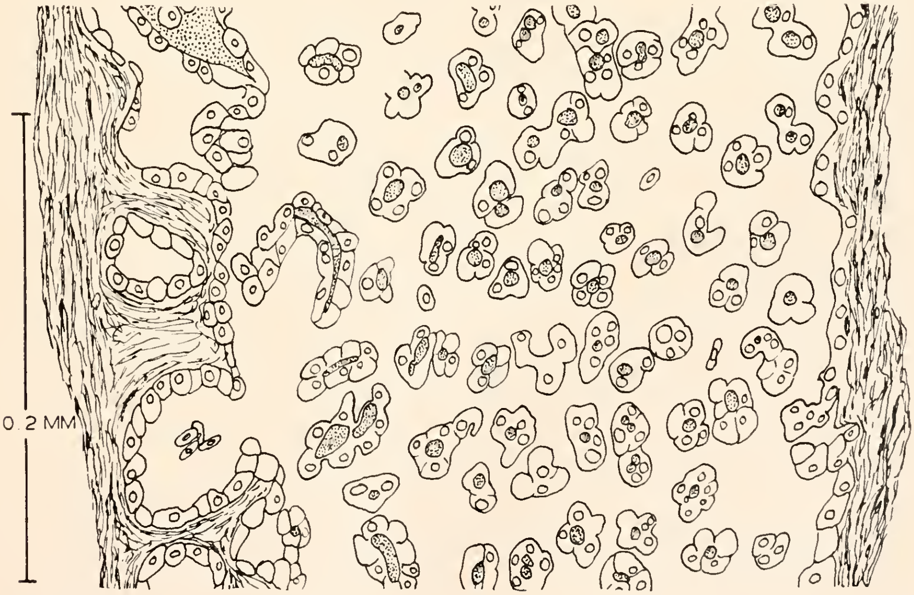

FIGURE 5. Columns of cavernous body. From a section perpendicular to the gill surface, parallel to the filaments. It is thus at right angles to the plane of section of Figures 2–4. Stippled area is the central core to which endothelial cells are attached; nuclei of the cells are shown as clear circles; pigment is not indicated. Openings on left are from basal arteries. Only a few red cells are included.

but the arterial muscle does not continue into the wall of the cavernous body. At the distal end of the cavernous body the wall is continuous with the double-layered sheet of connective tissue which forms the center of the filament. Figure 4 shows the exit from the cavernous body into a secondary lamella. The blood cells have been omitted for the sake of clarity, but all the blood spaces of the section were well

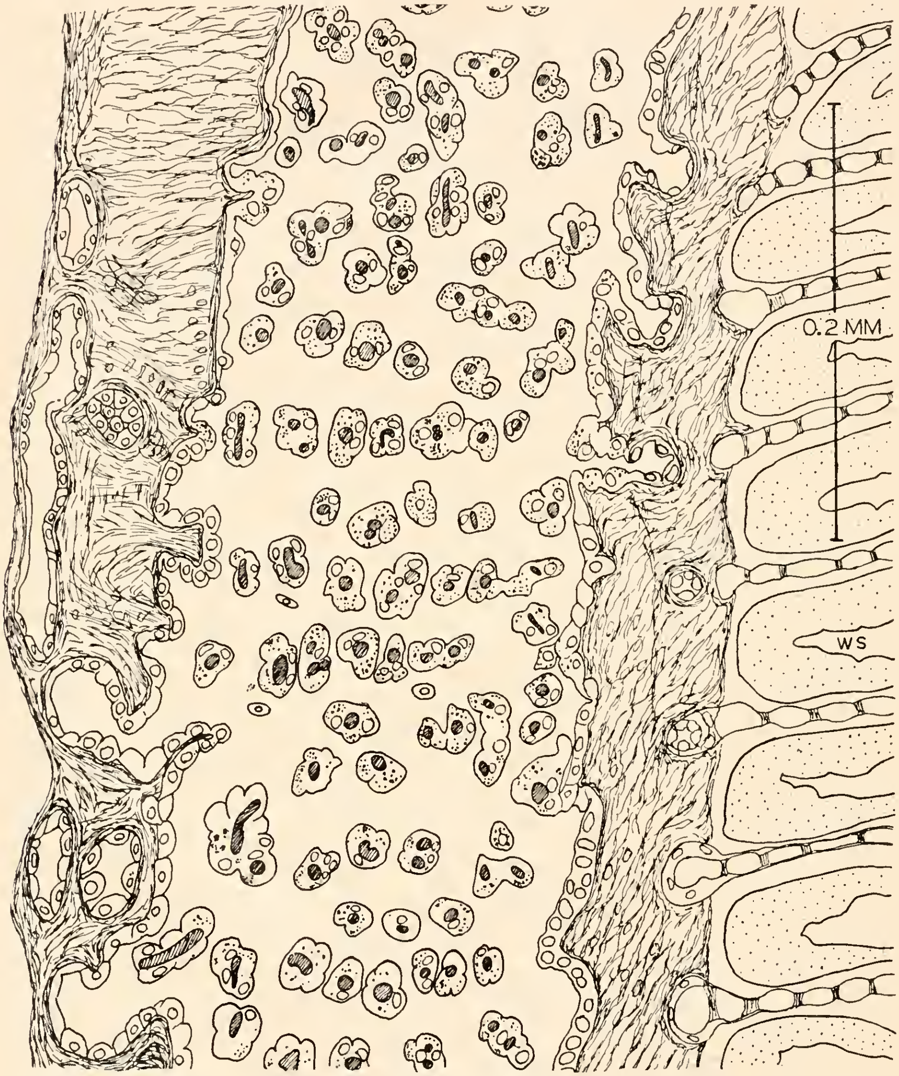

Figure 6. Columns of cavernous body. From a section perpendicular to the gill surface, parallel to the filaments. Central core of columns appears dark; pigment granules in endothelial cells are shown accurately. On left are openings into cavernous body from parallel artery; on right are openings from cavernous body into secondary lamellae. Epithelial layer is continuous with the surface of the lamellae and is indicated by stippling. Most of the red cells are omitted.

filled with erythrocytes. The connection to the other secondary lamella, lightly indicated here, appears two sections beyond this one.

The relationships are shown better in Figure 6. This is a drawing of a section parallel to the filament and perpendicular to the gill surface. Because the cut was made at a slight angle, there is a progression along the right wall. At the upper end is the exit from the lumen of the cavernous body; at the lower end is the entrance into the lamella proper. The space between the vascular channel and its overlying epithelia is present in all the sections but is presumed to be an artifact. The crypts and cavities of the opposite wall are connections between arteries and the cavity of the cavernous body. This becomes very clear when other sections of the series are examined.

From the scale which is included in the drawing it is clear that the number of secondary lamellae arising from the cavernous body can be considered to be approximately twenty pairs per millimeter. A similar value is reached when one counts the number of openings from arteries into the cavernous body.

The secondary lamellae

The lamellae, which were shown in Figure 5 taking their origin from the cavernous body, are thin structures which are shown diagrammatically in Figure 2, and as they actually appear in a cross section of a filament in Figure 3.

They are composed of a double sheet of epithelium which is seen well in Figure 7. This is a small part of the cross section through one lamella, the right end being the free edge of the sheet. In such a section the space between the epithelial layers, 0.01 to 0.02 mm in thickness, seems to be divided into a large number of channels containing red cells. It is these spaces which are referred to by many writers as capillaries. To be more accurate the apparent partitions are pilaster cells. Each seems to consist of a hollow tube with a fluted surface, composed of basement membrane which is continuous with that underlying the epithelium. The hollow tube contains the cell itself. This is compatible with the description of the ultrastructure of the teleost pilaster cells by Newstead (1967). The relationship is seen clearly in that part of the section where the epithelium is separated from its basement membrane. It also shows well in the bases of the secondary lamellae in Figure 6. Keys and Willmer (1932), in describing the pilaster cells of the eel, ascribed to them the function of keeping the two epithelial membranes apart, a concept which gave rise to their name. It would seem that their function is rather to hold the two epithelial layers together and prevent their being pushed apart by the pressure of the blood between them.

The essential difference between this arrangement and capillaries is seen more clearly from a different view. Figure 8 shows a section through a secondary lamella parallel to its surface. It is impossible to obtain more than a small part in a single section, due to its thinness. The upper right of the section is the distal artery, connected with the secondary lamella. One layer of epithelium is at the left, with the nuclei indicated; the other, cut at a long slant, is below with no cellular detail shown. The pilasters, now seen from their end, are stippled. A few red cells are included, but most of them are omitted for the sake of clarity. The distribution of pilasters is random, except near the outer edge of the lamella. Here they are somewhat enlarged and form a broken partition a short distance from

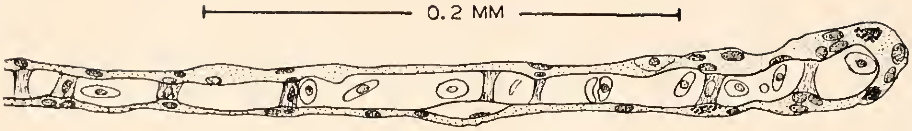

FIGURE 7. Structure of secondary lamella. Cross section through a secondary lamella near its free edge, which is at the right. The cavity is largest at the free edge. The apparent partitions are pilaster cells extending from one epithelium to the other.

the edge, a situation which results in the difference in appearance of the terminal space of a cross section of the lamella as seen in Figure 7. This is interpreted as a device which tends to spread the flow of blood more evenly across the cross-section area of the lamella.

The course of blood flow

From the information yielded by the injected animals, it is clear that the course of blood through the gills is as follows. Arising from the branches of the afferent branchial arteries, the basal artery carries the blood along the septum at the base of each filament, continuing to the very end of the filament. Along the outer surface of this artery there are approximately twenty openings per millimeter into the parallel cavernous body. Thus with a filament length of about 12 mm as shown in

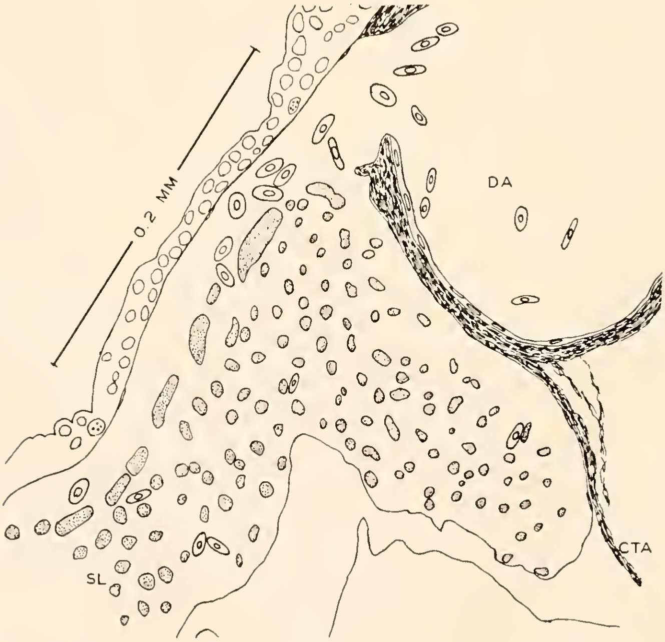

FIGURE 8. Structure of secondary lamella. Section very nearly parallel to the surface of the lamella. The pilasters, seen in end view, are indicated by stippling with details omitted. A few of the red cells are included for comparison with the available spaces. The epithelium covering the lamella was cut at a slant; the outline of the nuclei is indicated in the upper epithelium.

Figure 1, there would be approximately 240 openings from each artery into its cavernous body. Any cell could pass through any one of them. Within the cavernous body, which also extends the length of the filament, blood has a considerable "choice" of direction of flow. A cell which had entered could be carried in either direction along its length, and there is considerable freedom of movement in other planes. Along each millimeter of the cavernous body there are approximately 20 pairs of exits, each exit opening into a secondary lamella. The latter is so thin that there is little room for movement except in one plane, but within that restriction blood is free to move in any direction. Finally twenty pairs of openings per millimeter lead into the distal artery from secondary lamellae. The distal artery in turn delivers the blood to the collector branch of the efferent branchial artery.

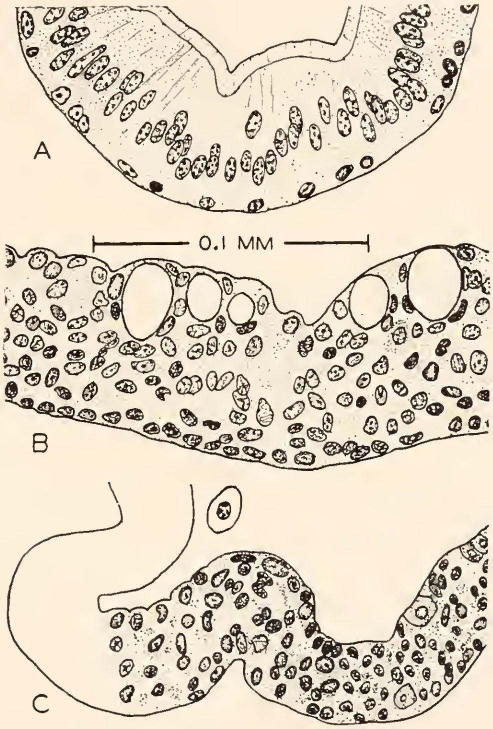

FIGURE 9. Epithelium of the expanded water channel. A. Small strip of the epithelium found in the basal half of the water channels of the anterior hemibranch. Outer surface of each cell is strikingly modified, either as a brush border or as cilia. B. Type of epithelium found in the distal half of the water channels of the anterior hemibranch. C. Type of epithelium found in all parts of the water channel of the posterior hemibranch. A brush border is never present. Mucous cells are also lacking.

Water channels

It is clear that water bathes the epithelium which covers the lamellae. Water is thus located between the lamellae, and between the free edges of the lamellae of one filament and those of the adjacent filament. Between the cavernous bodies the epithelium forms a wide-open channel, seen clearly in Figure 2. In the basal half of the anterior hemibranch the epithelium of this enlarged water channel is very regular, and the cells possess either a thick brush border or cilia (Fig. 9A). From our preparations one cannot decide which is present, but our interpretation leans toward a brush border. The distal half of the channel is quite different (Fig. 9B). The nuclei of the epithelium are randomly distributed, variable in size and staining capacity, and there are large numbers of mucous cells in different stages of development. Scattered larger cells with finely granular eosinophilic cytoplasm are interpreted as young mucous cells.

In the posterior hemibranch the corresponding spaces have a still different epithelium (Fig. 9C). There is never a brush border (or cilia); the epithelium is almost as thick but the nuclei are less regularly distributed, are more numerous, smaller and more deeply staining. There is an occasional larger eosinophilic cell with more lightly staining nucleus and clearly delimited finely granular cytoplasm. This type of epithelium lines the channel for its entire extent in the posterior hemibranch.

There is no information which demonstrates the course of water through these channels. We do not know the relative amounts which pass along the wide channel as compared with that between lamellae, or what fraction of the water passing through the gill pouch might move between the two hemibranchs without passing through any of the spaces indicated.

Discussion

Measurements of the difference in pressure between ventral and dorsal aortae show relatively little fall in pressure across the gills (Burger and Bradley, 1951). Fishman states (1967, page 216) that "Ingeniously, relatively little of this energy is lost as blood traverses the gills." The question arises how the blood can pass through the respiratory area without greater loss in pressure.

The morphological arrangements seem to offer a simple and clear explanation. It has been pointed out that blood from the basal artery is free to pass through many possible openings into the cavernous body; that within the cavernous body the blood is free to move in various directions and to exit through any one of many openings; that even within a lamella with its single entrance and exit, there is freedom of direction of movement among the pilaster cells. It is only when the blood has left the secondary lamella and has entered the distal artery that its flow again becomes restricted to one direction. With all this freedom of "choice" the blood will follow the course of least resistance. This is another way of saying that it will follow the line of least pressure drop, since the fall in pressure is due largely to overcoming resistance. Transient pile-ups of erythrocytes, which can be observed in the usual capillary circulation, may increase resistance to the point of markedly slowing or even stopping flow. In the blood channels of the gill such partial or complete blocking can have the effect only of diverting the flow to a course with less obstruction.

The arrangement of the blood in a sheet also results in a reduction of the area of contact between blood and wall, thus decreasing resistance. The area of contact in this arrangement is only about one half that which would prevail if the blood were contained in parallel tubes of the same diameter and the same total cross-sectional area.

Sheldon, Sheldon and Sheldon (1962) were surprised that the pulse wave persisted as the blood passed through the gill. They called attention to what they named the "arterial sinus" and which we have termed the cavernous body. They comment that this should be "ideally suited to damp out any pulse wave." However, they appear to have considered that the passageways through the structure were more tortuous than they really are, and that the core of the "trabeculae" consisted of smooth muscle. Figure 6 shows that the course of blood is not necessarily tortuous. The outer wall appears to have little elasticity and the core of the columns is probably non-elastic connective tissue and not smooth muscle. Furthermore a considerable degree of rigidity of the lamellae must result from the presence of very large numbers of pilaster cells. These factors, taken together, give reason for the persistence of the pulse wave through the gill. Stretching and recoil of the containing walls are required to absorb and damp out the pulse wave.

Another question which arises is how such a large amount of blood can pass through the gills, since as much must pass through these as through the entire systemic circulation. In our preparations there is no evidence of shunts between the afferent and efferent supply of the gills, which is in agreement with the findings of Sheldon, Sheldon and Sheldon. This study does not give morphological support for the statement of Robin and Murdaugh (1967) that "there is indubitable evidence that there is some degree (usually small) of pregill to postgill shunting" (page 236). Piiper and Shumann (1967), working with the dogfish *Scyliorhinus stellaris,* accept the difference in gill arrangement in elasmobranchs and teleosts. But in spite of these differences they also accept the presence of shunts between basal and distal arteries of the filament because such shunts are reported in the teleosts by Steen and Kruysse (1964). If there are morphological shunts in Squalus they are probably in the basal part of the gill, proximal to the base of the filaments. This possibility was not investigated. Certainly most of the blood is distributed through relatively large vessels (basal arteries and cavernous bodies). Then suddenly the blood is delivered to perhaps 30,000 parallel lamellae in each hemibranch. With 18 hemibranchs, the total dispersion through parallel channels is of the order of half a million. The individual blood cell, however, travels only a millimeter or two in traversing the lamella. The abrupt beginning and end of the finer channels, and their parallel arrangement, are well suited to provide the structural mechanism for allowing large amounts of blood to pass through the gills.

The course of blood through the lamellae is clear. The question arises whether the structure furnishes the possibility of a counter-current flow relative to the water. The arrangement of the gill is very different from that of the teleosts in which the counter-current flow was first described by van Dam (1938) and supported by the experiments of Hazelhoff and Evenhuis (1952).

With the known route of blood, to give a complete counter-current it would be necessary for the water to move into the space between the outer ends of the lamellae, and then pass inward between adjacent lamellae to the enlarged water channel lying between cavernous bodies. It is possible that this is the route taken by the water, but without further evidence this must remain dubious. The structure of the gills seems to fit better the "multicapillary" model suggested by Piiper and Schumann (1967). It becomes clear that more information is needed concerning the details of water flow. Robin and Murdaugh (1967) conclude on physiological grounds that the counter-current pattern does not fit their findings, and also that only 50% of the total inspired water seems to be effective in gas exchange. The various courses which water might follow may be of significance in relation to these findings.

One peculiarity which appears in our material is the fact that the diameter of the distal artery appears in sections to be markedly smaller than that of the basal artery. It is clear that all the blood entering through the basal artery must leave the filament through the distal one. It does not seem possible that the difference in diameters can exist to such a degree in the living state. This would cause a fall in the post-gill pressure greater than actually occurs. In distal arteries whose cross section is very small the thickness of the muscle layer is greater than that of the basal artery. However, when arteries of similar size are compared in the sections, the thickness of the muscle layer seems to be of the same order. While it could be argued that only those with the same amount of muscle show the same diameter in sections, it seems more probable that the different sizes in sections represent a difference in response to the process of fixation. It might mean only that the distal arteries are preserved in a more contracted state than the less accessible and more slowly fixed basal arteries. Satchell (1962) concluded that anoxia in *Squalus acanthias* causes a vasoconstriction at some point in the gills before the lamellae are reached. The basal arteries furnish a possible site. However, the potential contractility of the distal arteries must also be taken into consideration in the interpretation of vascular changes.

The presence of a well-formed brush border on the cells of the basal half of the water channels of the anterior hemibranch raises several questions and answers none. Why this should be found only in the basal half, and why there should be none in the posterior hemibranch, must at present remain unanswered. Available data do not furnish a clue to the function of these brush-border cells. The data on exchange between blood and sea water in this species demonstrate that a very impermeable membrane separates blood and water. For example, although the major part of the urea passing from the blood to the exterior does so through the gills rather than the kidneys (Smith, 1931; Goldstein and Forster, 1962) it must be remembered that the concentration gradient is extremely high, since the concentrations in blood and sea water are approximately 20 g/l and zero. In absolute values the gill membrane is nearly impermeable to urea. According to Boylan (1967), compared to the urinary bladder of the toad the gill is only $^{1}/_{12}$ to $^{1}/_{15}$ as permeable to water and sodium, and only $^{1}/_{35}$ as permeable to urea. While the rate of exchange of urea at the gill varies with temperature and deviation from normal blood levels, Boylan does not consider that the data indicate active transport of urea. In fact there is no conclusive evidence of active transport across this gill, despite the branchial elimination or absorption of many substances. For example, Horowicz and Burger (1968) found that the influx of sodium through the gills is of the same order as through the skin, but the greater surface of the gills gives a greater total influx by that route. Their data do not indicate whether there is active transport. There is little evidence at this time that presence of cells with a brush border is correlated with active transport across the gill.

SUMMARY

1. The course of blood through the gills is described.

2. Blood passes first into arteries which parallel the filaments and lie at their base.

3. Blood then passes into the overlying parallel cavernous bodies through any of the approximately 20 openings per millimeter.

4. The cavernous bodies have non-elastic walls and, passing across the lumen, non-elastic columns. Blood is free to flow in any direction through the cavernous bodies. Each cavernous body has approximately 20 pairs of exits per millimeter, each one opening into a secondary lamella.

5. The secondary lamella does not contain capillaries, but consists of two sheets of epithelium held together, with a constant space between them, by large numbers of pilaster cells. Within the single plane of this structure blood is free to move in a variety of directions between its entrance and its exit into the distal artery.

6. Within the freedom of movement provided by the basal artery, cavernous body and secondary lamella, blood is free to follow the line of least resistance. This results in its following the line of least pressure fall, and accounts for the small loss of pressure during transit of the gill.

7. The cavernous body appears to be rather rigid, and the large number of pilaster cells restricts the separation of the two epithelial layers and must impart a degree of rigidity to the walls of the vascular spaces. It is due to the consequent restriction of stretch and recoil that the pulse wave is relatively undamped and continues into the dorsal aorta.

8. The arrangement of blood vessels into myriads of parallel sheets which start and end abruptly provides the anatomical basis by which large amounts of blood are passed through a seemingly highly restricted area.

9. There is evidence that both the basal and distal arteries have sufficient muscle tissue to be potentially capable of some degree of contractility, a factor which should be taken into consideration relative to vascular changes.

10. There is no apparent explanation for the presence, in the basal half of the water passages of the anterior hemibranch, of an epithelium with what appears to be a highly developed brush border.

11. The anatomical arrangement of the gill seems to fit a multicapillary model better than a counter-current one. However more information is needed as to the details of water flow in relation to vascular structures before a definite conclusion can be reached.

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THE REPRODUCTIVE CYCLE OF *GORGONOCEPHALUS CARYI* (ECHINODERMATA; OPHIUROIDEA)[1]

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To the present time, there has been no detailed study of the reproductive cycle of any ophiuroid. Thorson (on *Ophiocten sericeum,* 1934) and Smith (on *Ophiothrix fragilis,* 1940) made brief observations on the ovarian histology at different times of the year and determined the breeding seasons for their respective species. All other determinations of breeding seasons for ophiuroids are based primarily on the times of the year when larvae appeared in the plankton or on direct observations of spawning. Boolootian (1966) prepared a summary graph of the known breeding seasons of ophiuroids; however, no references to Smith or Thorson are made. Most ophiuroids have a spawning season of from one to three months. The duration of known spawning seasons for ophiuroids ranges from one month (*e.g., Ophiothrix texturata,* Olsen, 1942; Mortensen and Lieberkind, 1928) to six months (*Amphiura filiformis,* Olsen, 1942; Mortensen and Lieberkind, 1928; and *Ophiothrix fragilis,* Smith, 1940). *Ophiocten sericeum* spawns for 4 to 5 months (Thorson, 1934). The viviparous ophiuroid *Amphipholis squamata* appears to breed throughout the year (Fell, 1946).

Most studies of echinoderm reproductive cycles utilize the gonad index as the measurement of reproductive condition. The gonad index is defined as the ratio of the gonad volume to total wet weight × 100 (Lasker and Giese, 1954). This gives a useful measure of the relative size of the gonads, but it gives no indication of the actual condition of the gametes. Moreover, a gonad index cannot be determined on animals such as *Gorgonocephalus* where the numerous gonads fill most of the body cavity, and their attachments are such that they cannot be removed in their entirety from the animal. Only a few studies have employed periodic histological examination of the gonads as a means of determining the reproductive cycle in echinoderms (Yoshida, on *Diadema,* 1952; Tanaka, on *Stichopus,* 1958; Fuji, 1960a, 1960b, on *Strongylocentrotus nudus* and *S. intermedius;* Pearse, on *Odontaster,* 1965; Chia, on *Leptasterias,* 1964, 1968; Holland, on *Stylocidaris,* 1967). The need for information concerning the reproductive cycles of ophiuroids and the paucity of histological data on the reproductive cycles of echinoderms prompted this study.

MATERIALS AND METHODS

Monthly collections of *Gorgonocephalus caryi* were made for 13 months (from August 23, 1965, to August 26, 1966) from Boundary Pass in the San Juan Islands

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48° 44.4′ N., 23° 1.7′ W) from depths of 20–70 fathoms. Samples of the ventral gonadal lobes (Patent, 1968) were taken from 8 to 28 animals each month. The proportion of males to females varied from month to month, but the overall number of animals of each sex examined was about equal. The tissues were fixed in Heidenhain's susa fixative made up with sea water, dehydrated by the tertiary butyl alcohol technique (Johansen, 1940), embedded in Paraplast, and sectioned at 5 μ–7 μ. At least 20 gonad sections were made from each animal, and these were stained with a modified Masson's trichrome (Patent, 1968). The mean diameters of twenty of the largest oocytes (sectioned through the nucleolus) from each female were measured by averaging two diameters taken perpendicular to each other. The twenty oocyte diameters from each animal were averaged and the standard error determined. The standard errors were very small (from 0.8 μ to 2.9 μ). The average values for the animals collected each month were then averaged, giving a mean size for the largest oocytes for each month.

After examination of slides of gonads from all times of the year, the reproductive cycles of the female and the male were divided into five stages. These stages were derived independently for the male and the female.

The Maturity Index (M.I.) of Yoshida (1952) was calculated for both males and females for each month. The formula used is as follows:

$$\text{M.I.} = \frac{\Sigma[1\ (\#\ \text{animals in Stage 1}) + 2\ (\#\ \text{animals in Stage 2}) \cdots + n\ (\#\ \text{animals in Stage n})]}{\text{Total number of animals staged that month}}$$

RESULTS AND DISCUSSION

The stages of the reproductive cycle

Examination of the histological material showed that the reproductive cycle of the female and that of the male could be divided into five fairly distinct stages. The key criteria used are as follows:

Stage 1: Post-spawning. Females: The lumen of the ovary is filled with degenerating oocytes (Fig. 1A). Males: Spermatogenesis has stopped, and only spermatogonia and spermatozoa are present. There are often areas of the testicular lining which are devoid of spermatogonia (Fig. 2A).

Stage 2: Early growth. Females: Little or no post-spawning debris is present. The ooplasm stains predominantly red with Masson's trichrome (Fig. 1B). Males: the testes are small and the distribution of the spermatogonia is uneven. There are more spermatogonia near the end of the gonad where the germ cells enter and/or the gonads are very small with very few sperm present (Fig. 2B).

Stage 3: Resting. Females: Green granules can be seen as clumps in the ooplasm in stained material. The oocytes are under 121 μ in diameter (Fig. 1C). Males: The spermatogonia are evenly distributed, and there are deep indentations in the wall of the testis. In some testes, spermatogenesis has begun (Fig. 2C).

Stage 4: Late growth. Females: The largest oocytes are over 121 μ in diameter, and the outer membrane (Patent, 1968) has begun to form (Fig. 1D). Males: The layer of spermatogenic tissue is very thick and spermatogenesis is well under way. The spermatozoa do not yet form a separate mass in the testicular lumen (Fig. 2D).

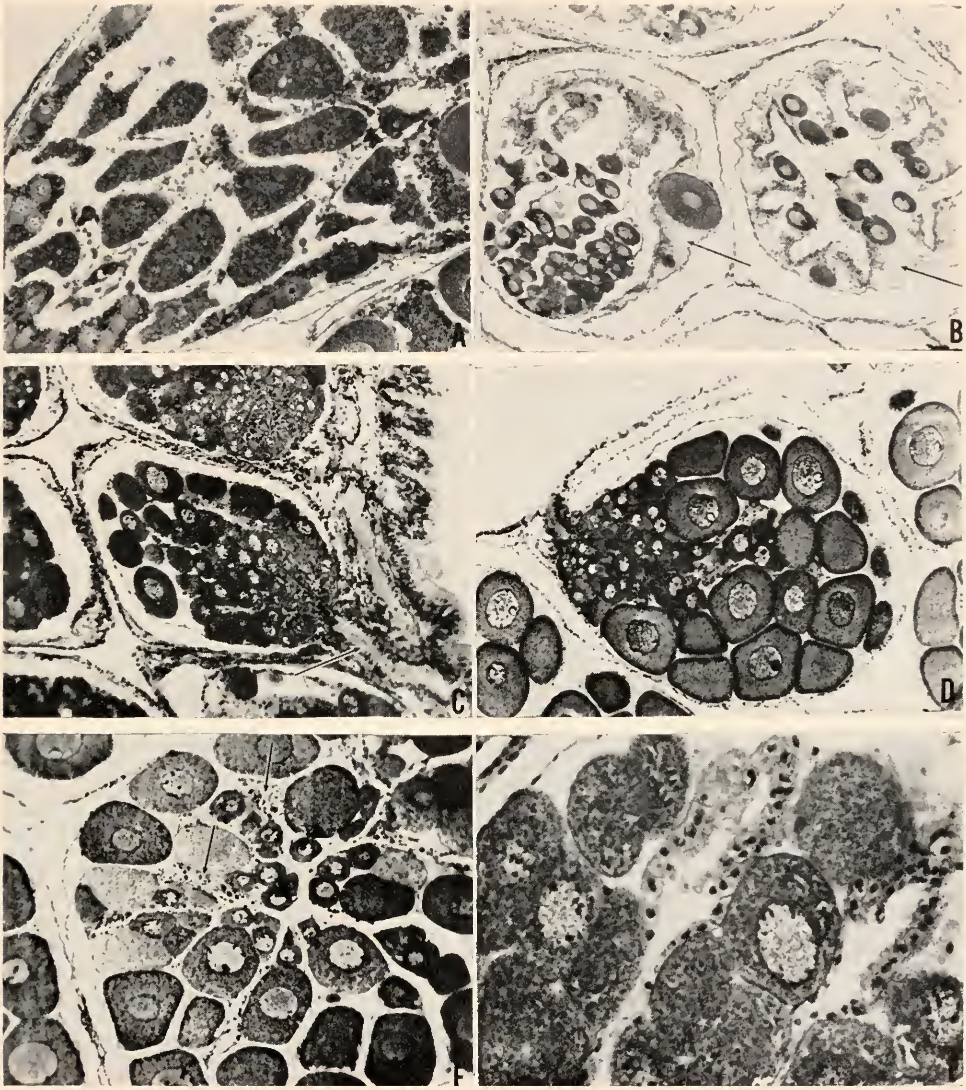

FIGURE 1. Histological sections of gonads of *Gorgonocephalus*. (A) Stage 1 ovary from animal collected in November. The lumen of the ovary is filled with degenerating oocytes. (B) Stage 2 ovaries from animal collected in December. The walls of the ovaries are still stretched, and the hemal fluid can be seen (arrows). Note the one large oocyte which was not released into the lumen when the animal spawned. (C) Stage 3 ovary. Note the uniform staining of the oocytes. The ovarian duct can also be seen (arrow). (D) Stage 4 ovary. The largest oocytes are located in the distal portion of the gonad. Note the lighter staining of the large oocytes (green with Masson's trichrome) and the dark staining of the periphery of the large oocytes (red with Masson's). (E) Stage 5 ovary. The oocytes are large, and debris left over from unspawned oocytes can be seen in the ovarian lumen (arrows). (F) Portion of an ovary from an animal collected in December. The layer of nurse cells lining the lumen is prominent. Clumps of material, some of which look like pycnotic nuclei of nurse or follicle cells can be seen inside the oocytes. Photographs 1A–1E are all 78 ×. Photograph 1F is 250 ×.

Stage 5: Spawning. Animals in this stage are assumed to have spawned at least once and will spawn at least once more before the spawning period is over (see below). Females: Cellular debris from the breakdown of unspawned oocytes is seen in the ovarian lumen (Fig. 1E). Males: A large number of sperm have been produced and are seen as a mass in the center of the gonad, separate from the layer of spermatogenic tissue (Fig. 2E).

The above criteria were used to classify all the animals from which gonadal tissue had been collected. However, much more can be said about the histology of the gonads, especially of the ovaries, at the different stages.

The ovarian cycle

In early Stage 1 ovaries, the degenerating oocytes are seen as distinct masses of material in the ovarian lumen (Fig. 1A). Later, the degenerating cytoplasm coalesces. Throughout Stage 1, spheres from degenerating oocytes are seen in the cytoplasm of the nurse cells which border the lumen and are attached to the young oocytes (Patent, 1968). It is difficult to measure oocytes in Stage 1 ovaries because the ovarian wall is still distended and very few oocytes are seen in a given section. In addition, the oocyte boundaries are indistinct.

In early Stage 2 ovaries, the walls are still stretched, so that the lumen and the fluid in the hemal space surrounding the gonad (Patent, 1968) are easily seen (Fig. 1B). Later, the walls contract and the gonad is more compact. The nurse cells are abundant and are frequently arranged as an epithelium lining the ovarian lumen (Fig. 1F). The oocytes often contain ingested cellular debris (Fig. 1F) (Patent, 1968). All oocytes in Stage 2 ovaries are under 80 μ in diameter.

The nurse cells in Stage 3 ovaries are more clumped. The largest oocytes have begun vitellogenesis. The cortical granules have also begun to form (Patent, 1968). The oocytes are more crowded and, instead of having a more or less oval outline as in Stage 2, they are developing a more polygonal form. The largest oocytes in Stage 3 ovaries range from 84 μ to 121 μ in diameter.

In the stage of late growth (Stage 4), the largest oocytes are from 122 μ to 150 μ in diameter. The outer membrane has not formed around all the large oocytes. In some ovaries, many oocytes will be developing the membrane, whereas it may not be present on any of the oocytes in other ovaries. The oocytes are packed tightly, and their shape is variable (Fig. 1D). The stalk attaching the oocyte to the nurse cells (Patent, 1968) is very evident. The cortical granules lie mostly at the periphery of the oocyte.

The size of the oocytes in Stage 5 ovaries is variable, depending on when the animals spawned and also probably on the size of the animal. In most Stage 5 animals, the largest oocytes were between 150 μ and 160 μ in diameter, with a range of 115 μ to 177 μ. Since fresh, living fertilized eggs measure 220 μ, it seems probable that fixation (in susa fixative) and dehydration (through the tertiary butyl alcohol series) cause about 20% shrinkage of the oocytes.

The histological condition of the largest oocytes is variable in Stage 5 ovaries. Usually, the germinal vesicle is similar to that of Stage 4 oocytes in size, but sometimes it has swollen (Fig. 2F). In two females collected in November, 1965, there were secondary oocytes present. The follicle cells around them were disintegrating,

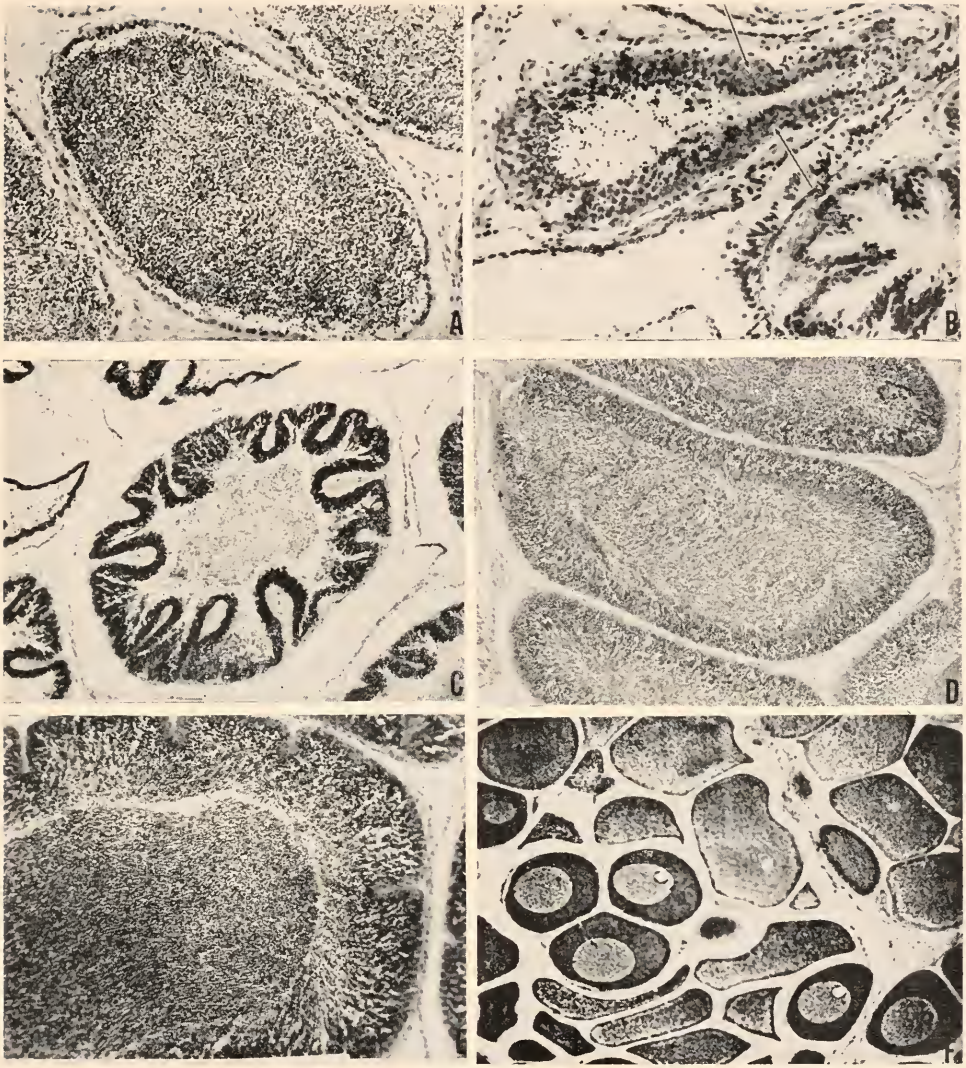

FIGURE 2. Histological sections of gonads of *Gorgonocephalus*. (A) Stage 1 testis. Spermatogenesis has stopped. Areas without spermatogonia can be seen along the testicular wall. 156 ×. (B) Stage 2 testis. Note the thick layer of spermatogonia at the duct end of the testis (arrows). 156 ×. (C) Stage 3 testis. The layer of spermatogenic tissue is thickened. The loops of germinal tissue underlain by hemal fluid in the hemal space are easily seen. 78 ×. (D) Stage 4 testis. The spermatozoa do not form a separate mass in the lumen. 62 ×. (E) Stage 5 testis, showing the separate mass of spermatozoa in the lumen. 78 ×. (F) Section of ovaries just before spawning, showing the shape of the eggs and their small nuclei. Primary oocytes with swollen germinal vesicles can also be seen, and the degeneration of the follicle cells is evident. 78 ×.

and their shape was distorted. The nuclei were small, and the cortical granules were all at the periphery of the oocytes (Fig. 2F). Since only two such animals were collected, it is probable that the oocytes undergo at least the first maturation division within hours of spawning. The gonads of these animals were fixed in the afternoon. When spawning occurred in the laboratory in the winter of 1966, it always occurred between 7:00 and 9:00 p.m. It is probable that the animals which contained secondary oocytes would have spawned the evening of the day they were collected, a few hours after the oocytes underwent maturation division. This is in keeping with the pattern in other echinoderms which have been studied, with the exception of euechinoids. Holland (1967) has concluded that the lack of accumulation of ripe eggs is a primitive echinoderm trait which has been altered in the euechinoids. In asteroids and some ophiuroids, maturation divisions do not occur until after spawning (Costello, Davidson, Eggers, Fox and Henley, 1957). In the cidaroid sea urchin *Stylocidaris affinis* (Holland, 1967) and in the crinoid *Comanthus japonica* (Dan, 1952), maturation divisions occur a short time

TABLE I

*Numbers of animals in each gonadal stage collected each month**

Females Stage no.	Year												
	1965					1966							
	Month												
	A	S	O	N	D	J	F	M	A	M	J	J	A
1			1	3	1								
2					3	1	1			1			
3						6	9	8	7	2	1		
4	4									8	3	2	3
5	9	10	12	3							1	1	4
Total	13	10	13	6	4	7	10	8	7	11	5	3	7
M.I.	4.7	5	4.7	3	1.8	2.9	2.9	3	3	3.6	4	4.3	4.6
Males Stage no.													
1				9	2								
2					7	2	3	3	1	2			
3					1	3	6	8	3	3			
4										3	1		
5	9	12	11	5						2	5	4	4
Total	9	12	11	14	10	5	9	11	4	10	6	4	4
M.I.	5	5	5	2.4	1.9	2.6	2.7	2.7	2.7	3.5	4	5	5

* M.I. = Maturity Index. The collections were made from August, 1965 to August, 1966.

before spawning. In *Comanthus*, all the animals spawn within a few minutes of one another during late afternoon in early October, and the oocytes undergo maturation division almost simultaneously throughout the population. Thus, it was possible for Dan to study the changes in the oocytes which precede and accompany maturation division. In *Gorgonocephalus*, the oocytes do not undergo maturation division simultaneously; rather, different stages can be seen at once in an ovary. As in *Comanthus*, the germinal vesicle is first drawn out towards the stalk connecting the oocyte with the other ovarian tissue. The nucleoli at this stage are fragmenting. It was not possible to see in *Gorgonocephalus* just how the oocytes broke away from their attachments, but the follicle cells are seen to be disintegrating. The oocytes of *Comanthus* and *Gorgonocephalus* both lose their shape after becoming free (Fig. 2F). The membrane of the germinal vesicle disappears and the spindle-forming bodies migrate to one pole. Dan wrote that this was the stalk end in *Comanthus*, but this could not be determined for *Gorgonocephalus*. After the spindle-forming bodies migrate, maturation division begins.

As is seen in Table I, some animals of both sexes were in the spawning stage from August to November, 1965, and from June to August, 1966. Assuming that the spawning season at a given locality is fairly constant from year to year, *Gorgonocephalus* has a spawning season of six months. This also assumes that the criteria used for determining when spawning is taking place are valid. The key criterion for determining the spawning season of the females was the presence of cellular debris in the lumen of the gonad. It was assumed that this debris represented the remains of oocytes which had matured but which remained in the ovary and were not spawned. Pearse (1965) and Delavault (1961) noted that degenerating oocytes were found in some asteroid ovaries throughout the year. Pearse postulated that these oocytes serve as storage organs for the nutrition of other oocytes. The debris in the *Gorgonocephalus* ovary (assumed to be the remains of unspawned oocytes) could represent the remains of such storage oocytes which had been broken down. However, the evidence is to the contrary. In *Gorgonocephalus*, a few large residual oocytes occasionally remain in the ovary after the spawning season (Fig. 1B). These are attacked by phagocytes *in situ* some time prior to the next spawning season, and no fragments are released into the ovarian lumen. Secondly, the debris in the lumen of the gonad is found only during the presumed spawning period and in the post-spawning period, not year around. Thirdly, the first appearance of the cellular debris coincides with the first time that oocytes over 150 μ in diameter are seen in the ovaries. These large oocytes do not appear in the one animal collected that month which is assumed to have spawned (*i.e.*, the ovarian lumina contained cellular debris). Lastly, the criteria for determining the stages of the reproductive cycle for males and females were arrived at independently, yet the data from the males also indicates that the animals spawn from June through November. Thus, it seems reasonable to conclude that *Gorgonocephalus* spawns for six months of the year and that the debris in the ovarian lumina is derived from unspawned oocytes.

Other workers (Tanaka, 1958; Chia, 1964, 1968) who have divided the reproductive cycle of echinoderms into stages have included a resting stage. In *Stichopus*, most of the females appear to have a post-spawning rest period of three months (Tanaka, 1958). This is usually assumed to be the typical echinoderm pattern

(Boolootian, 1966). Chia (1964, 1968) has shown that in *Leptasterias* there is a resting period of several months after the largest oocytes have reached their terminal size. During this time there is very slow growth of the small oocytes. In *Gorgonocephalus,* there is a resting period in the females from January through March. During this time, the average diameter of the largest oocytes remains relatively constant, and the ovaries of animals collected in January show no striking differences from those collected in March. However, it is possible that more oocytes are growing and reaching the maximum size for this stage (under 121 μ). The resting period occurs after the oocytes have undergone considerable growth (from a maximum diameter of 78 μ in December to a maximum of 104 μ in Jan-

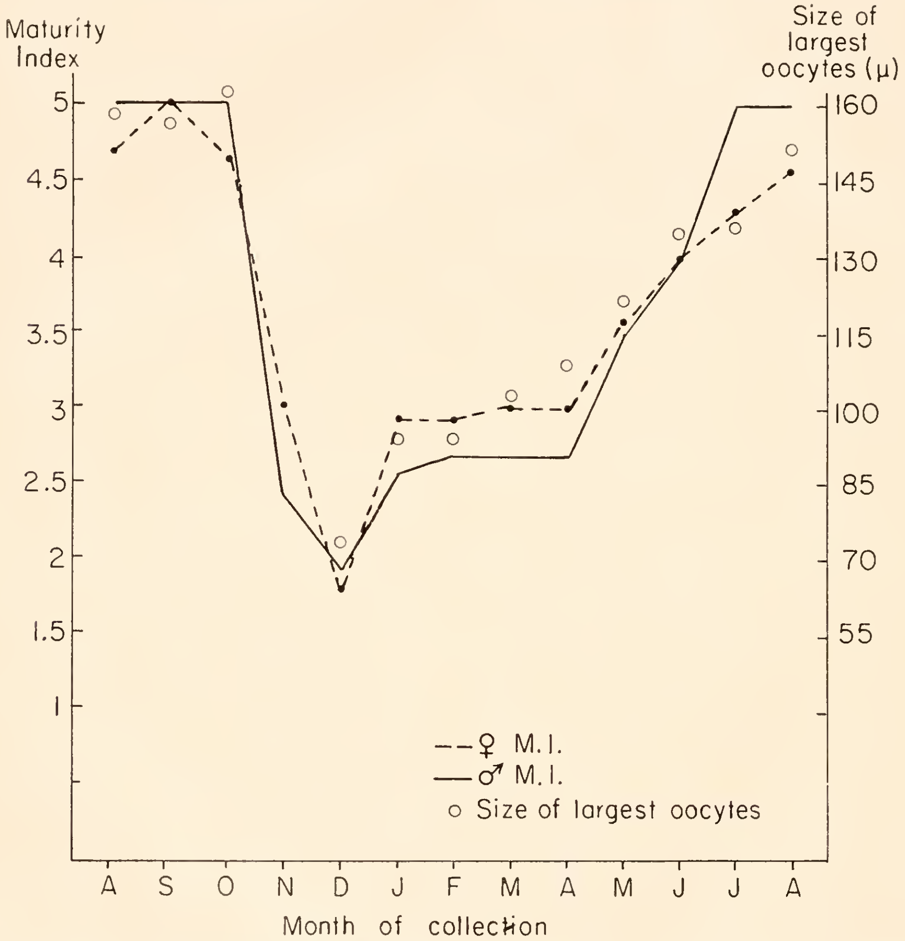

FIGURE 3. Graph showing the Maturity Index (left scale) and the size of the largest oocytes (right scale) for the animals sampled in this study. The first month of collection was August, 1965; the last month was August, 1966. All collections were made in Boundary Pass in the San Juan Islands.

uary). Probably the growth from December to January and what little growth may occur during the resting period result from the utilization of the nutrients derived from the degenerating unspawned oocytes at the end of the spawning season. The rest period from January through March then corresponds to the period of least availability of nutrients. During the winter months in the San Juan Islands, there is very little plankton. By April, the daylight hours have increased considerably, and the plankton becomes more abundant. In April, the oocytes resume growth. Holland (1964) has shown that in *Strongylocentrotus purpuratus* continuous feeding stimulates, and starvation inhibits, production of large cells in the ovaries (and of new cells in the males). Thus, at least in a regular echinoid, the availability of nutrients can be reflected in the development of the gonads. However, whether the rest period in *Gorgonocephalus* results directly from a lack of food or whether it is controlled in some other way and evolved in response to the period of minimum food availability cannot be stated.

It can be seen in Figure 3 that the diameters of the largest oocytes correlate well with the Maturity Indices for the different months. The only time that oocyte diameters were used to determine the stage of an animal's ovaries was when there was some question as to whether an animal was in Stage 3 or 4; occasionally it was difficult to determine if the outer membrane had begun to form. In these cases, the animals were placed in Stage 3 if the oocytes were under 121 μ in diameter and in Stage 4 if they were over 121 μ. This was necessary in only a few cases; so in general, the average diameter of the largest oocytes is a good measure of gonadal maturity.

The testicular cycle

Although the ovarian stages used in this study were determined independently of other studies, the testicular stages are very similar to those reported by Tanaka (1958). He divided the reproductive cycle of *Stichopus* into five stages: Resting, recovery, growing, mature, and shedding. Histologically, the resting stage of *Stichopus* testes resembles the post-spawning stage of *Gorgonocephalus*. However, as is seen in Table I, this post-spawning stage is not a resting stage for male *Gorgonocephalus*, as it lasts no more than a month. The recovery stage of *Stichopus* is similar to the early growth stage of *Gorgonocephalus* males, and the growth stage of *Stichopus* is almost identical histologically to what is designated as the resting stage (Stage 3) in *Gorgonocephalus*. However, some male specimens of *Gorgonocephalus* appear to rest in Stage 2. At least, during the months of January through April, the proportion of animals in Stages 2 and 3 remain stable for the population, as is shown by the Maturity Index (Table I, Fig. 3). One could conclude that the population shows a resting period from January to April, but that one cannot define a resting period precisely for individual animals.

General comments

The Maturity Indices for the monthly collections are plotted in Figure 3. The Maturity Index (Yoshida, 1952) is useful when the sample size from different months is different, and it provides a way to compare the data from males and females. It can be seen (Fig. 3) that the criteria used for determining the male and female cycles are almost equivalent. The differences between the males and females could be due to imperfections in the method of estimating the stage of development or to differences in the ways in which gametogenesis proceeds in males and females. If one assumes no defect in the method, some comments can be made about the differences. Table I shows that males in Stages 2 and 3 are present from January through May, whereas Stage 2 females are no longer present after February, except for one aberrant individual in May. Stage 4 males are present for a short time only, in May and June, whereas there are Stage 4 females present from May through August. This could indicate that intensive spermatogenesis does not begin until just before the spawning season and that once it has begun, all the males are spawning. In contrast, oogenesis proceeds for a longer time before the oocytes reach spawning size. Not all the females spawn throughout the season, but it cannot be determined how frequently a particular female spawns during a season. Females spawn more than once, most of them through October, some into November.

In the winter of 1966–67, specimens of *Gorgonocephalus* collected southwest of the monthly collection site spawned many times in the laboratory. All spawnings took place between 7 and 9 p.m. Spawning between 8 and 10 p.m. has been noted in *Ophiura brevispina, Ophiopholis aculeata,* and *Ophiocoma echinata* (Grave, 1899). Only once was the spawning of *Gorgonocephalus* observed closely (by Dr. Gregory Patent). The animal observed was a male which spawned for about ½ hour, pumping the disk up and down. Sperm were released on the downward push. The dates on which spawning occurred were as follows: November 21, 1966; December 20, 1966; January 7, 15, 16, 17, and 21, 1967; February 10 and 21, 1967; and March 10, 1967. It is not known whether the same or different animals spawned on the different days. Sometimes only males spawned; at other times both males and females spawned. No pattern can be seen in the days on which spawning occurred. February 10 was the last day on which females single males which had been in the laboratory for at least two months would not spawned, so it is possible that the later February and the March spawnings by have occurred in the field. Even so, the animals from this location spawned three months later than the animals from Boundary Pass would have spawned the previous year. It is possible that the spawning season of *Gorgonocephalus* varies from year to year. Giese (1959) followed the reproductive cycle of *S. purpuratus* from one location for many years and found that the gonad index curve and the time of spawning were often very different from one year to the next. The factors causing this variation are unknown. It is also possible that specimens of *Gorgonocephalus* from different locations have different spawning seasons. Thorson (1934) found that *Ophiocten sericeum* from different locations around East Greenland varied in the stage of ovarian development. Further study is required to determine the cause of the observed differences in spawning season of *Gorgonocephalus* from different localities.

I wish to thank Dr. Robert Fernald, director of the Friday Harbor Laboratories, for his generous provision of facilities, and Dr. Cadet Hand for his guidance throughout this study.

SUMMARY

1. Samples of gonads from *Gorgonocephalus caryi* collected monthly over a 13-month period were examined histologically to determine the course of the reproductive cycle.

2. The reproductive cycle of both sexes was divided into 5 stages: Post-spawning, Early Growth, Resting, Late Growth, and Spawning. The Maturity Index (Yoshida, 1952) was calculated separately for males and females for each month.

3. The histology of the ovaries in the various stages is described.

4. Maturation divisions in females probably occur on the day of spawning and proceed very much as in the crinoid, *Comanthus japonica* (Dan, 1952).

5. *Gorgonocephalus* has an annual reproductive cycle and spawns for six months of the year, from June through November in the population studied in 1965–1966. In the winter of 1966–1967, animals collected from a different location spawned in the laboratory from November through March.

6. Each animal spawns more than once during the spawning season.

7. Gonad development is arrested after the initial stages of gametogenesis have commenced rather than immediately after spawning, as occurs in most other echinoderms.

8. The curve for growth of the largest oocytes corresponds well with the female Maturity Index, indicating that oocyte size is a good measure of gonadal maturity.

9. The male and female Maturity Indices correspond well, and the differences between them can be explained by differences between oogenesis and spermatogenesis.

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SENSE ORGANS ON THE ANTENNA OF A PARASITIC WASP, *NASONIA VITRIPENNIS* (HYMENOPTERA, PTEROMALIDAE)[1]

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One of the objectives of the series of studies on the sense organs of insect antennae undertaken by the writer is to discover whether insects occur that lack chemoreceptors of a particular type or, conversely, whether any kind is universally present. Thin-walled chemoreceptors with many fine pores in their cuticle, where filaments from the olfactory dendrites end, have been found in every species so far examined intensively (Slifer, 1967 review; 1968a, 1968b; Myers, 1968). Their cuticular parts take the form of hairs, pegs or plates that are domed, raised or flattened. Similarly, thick-walled chemoreceptors have been identified in each of the species listed in the references given above. These consist of hairs, pegs or bristles and have a single pore at the tip where the dendrites are exposed to the air. Some arise from the surface of the antenna while others, usually pegs, are set in cavities where the antennal cuticle is invaginated so that the tip of the peg lies below and sometimes at a considerable distance from the surface of the antenna.

A preliminary examination of the antennae of *Nasonia vitripennis* suggested that this was a species that lacks thick-walled chemoreceptors although thin-walled chemoreceptors are present in abundance. It was not until the study was nearing completion that a few thick-walled chemoreceptors were found.

MATERIALS AND METHODS

Samples of adult *Nasonia vitripennis* (Walker) from wild type and scarlet stocks were kindly given the author by Dr. Anna R. Whiting who is now at the Oak Ridge National Laboratory in Tennessee. [*Nasonia vitripennis* (Walker) is also known as *Mormoniella vitripennis* (Walker) and the latter name is frequently used in current literature.] Some of these were fixed in Bouin's solution and others in 5% formalin.

Some antennae were stained in borax carmine or with the Feulgen technique and mounted whole. Others were examined in glycerol which usually makes pores in the thin-walled receptors easier to see. Entire insects were treated with a 0.5% solution of crystal violet for the identification of pores in the cuticle of sense organs on the antennae and elsewhere (Slifer, 1960). Males of the scarlet stock used have pale antennae and it is easier to study whole mounts made with them than with dark wild type antennae. Antennal flagellae were embedded in Paraplast, sectioned at 5 or 7 μ and stained with Holmes' silver method, Mallory's stain or Heidenhain's iron-hematoxylin.

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RESULTS AND DISCUSSION

Nasonia vitripennis is an insect of economic importance since its larvae destroy the pupae of Diptera by developing within them and of scientific interest because it has been much used in laboratories for studies in genetics and related fields. A valuable review, listing over 200 references to earlier work on the species, has been published by Whiting (1967).

The antenna of the female *Nasonia* was described by Jacobi (1939). In general, the present paper confirms his results but information and techniques that were not available thirty years ago now permit a more detailed description and better understanding of the function of the various sense organs. The male antenna is also included.

In both sexes the antenna consists of a long scape, a shorter pedicel and a flagellum composed of twelve subsegments (Fig. 1). The first and second flagellar subsegments are short and, like the scape and pedicel, provided only with hairs believed to be tactile. Olfactory receptors, together with a few tactile hairs, are numerous on the remaining subsegments. Subsegments three to eight are each constricted sharply at their distal and proximal ends. Subsegments nine to twelve, in contrast, form a compact unit and lack deep constrictions between them although their boundaries are clear. The twelfth subsegment is very small (Figs. 1, 3) and, instead of being placed symmetrically at the apex of the antenna, the greater part of it faces medially and ventrally. Although *Nasonia* males are distinctly smaller than are the females the antennal flagellum is nearly the same length in both sexes —about 350 μ for the material examined here.

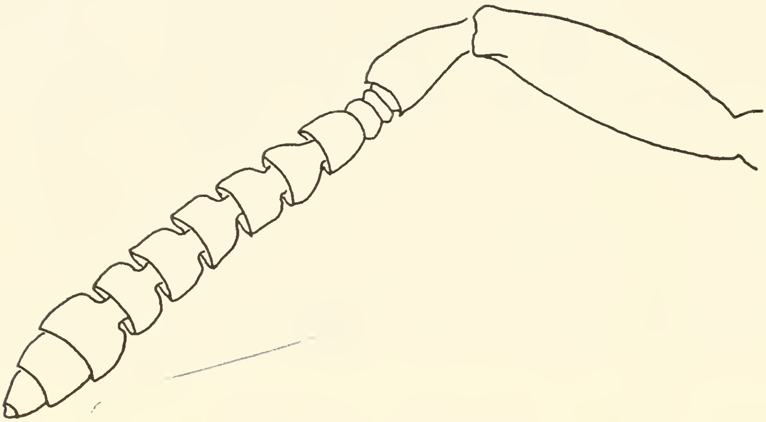

FIGURE 1. Right antenna of male as seen from medial surface. Scape (right), pedicel and flagellum with twelve subsegments. Sense organs omitted. × 168.

Five structurally different types of sense organs occur on the flagellum of both males and females (Fig. 2). Two of these are almost certainly tactile in function and the other three have the characteristics of chemoreceptors. Coeloconic and ampullaceous sense organs are commonly present on the antennae of Hymenoptera but none were found in *Nasonia* by Jacobi (1939) nor by the present writer.

Tactile hairs

Although these are very small and it is impossible to see with the light microscope that they possess all of the structural features of tactile organs proven to be such in other species, enough evidence can be obtained to assign this function with a high degree of certainty. They are of two kinds. The first (Figs. 2a, 3e) is slender, nearly straight, sharp-tipped, about 12 μ long and has a basal diameter close to 0.5 μ. It was described by Jacobi (1939) as a *kleines Haar* that is probably tactile. His drawing of a longitudinal section through the hair shows only its outline with an indistinct strand of material entering its base. When the intact insect is immersed in a solution of crystal violet the dye does not enter these hairs. They are present on each of the flagellar subsegments except the twelfth. The same type of hair, usually larger and stouter, occurs on the scape, pedicel and other parts of the body. Few satisfactory sections that included both one of these hairs and the cells associated with it were obtained. Sometimes a nerve strand could be seen extending below the hair base and ending in a group of three or four cells. This strand is so exceedingly thin that it is improbable that it consists of more than one dendrite from a single sensory cell. A tactile receptor, as is well known, is usually innervated by one neuron. The other two or three cells are probably sheath cells.

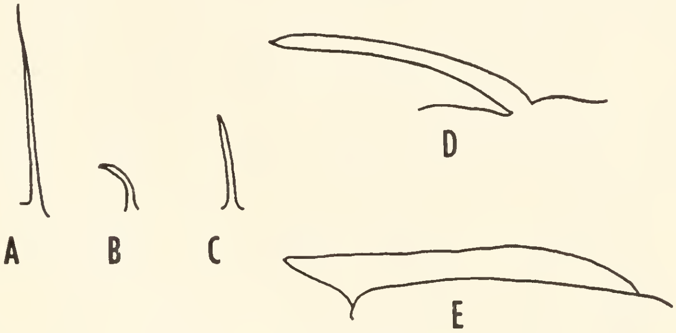

FIGURE 2. Five types of sense organs found on flagellum. All drawn in lateral view and with surface directed toward distal end of antenna at left. A, slender hair with sharp tip, probably tactile; B, curved hair, probably tactile, from twelfth subsegment; C, thick-walled chemoreceptor with opening at tip from twelfth subsegment; D, thin-walled chemoreceptor; E, plate organ. × 1452.

Jacobi (1939, Figs. 28, 29) found two kinds of hairs, both small and thin-walled, on the twelfth subsegment. He suggests that both are tactile. The smaller of the two probably does have that function. It is sharply curved, from 5 to 7 μ long and has a basal diameter of 0.5 μ (Figs. 2b, 3b). The tip is slender but not so sharp as that of the hair described in the preceding paragraph. The hairs do not stain when the insect is treated with crystal violet and this indicates that they lack pores where olfactory dendrites are exposed. The cellular parts of these receptors could not be distinguished from those of the other sense organs close to them. According to Jacobi (1939), the female beats or drums rapidly with the antennal tips on the dipteran pupal shell when hunting for a suitable place to lay eggs. These small curved hairs on the end of the twelfth subsegment would be ideally located and constructed to serve as tactile organs for distinguishing differences in the pupal covering. In the male, contact of the antenna with the female is important in courtship (Barrass, 1960) ; but whether the antennal tip is especially concerned is not known.

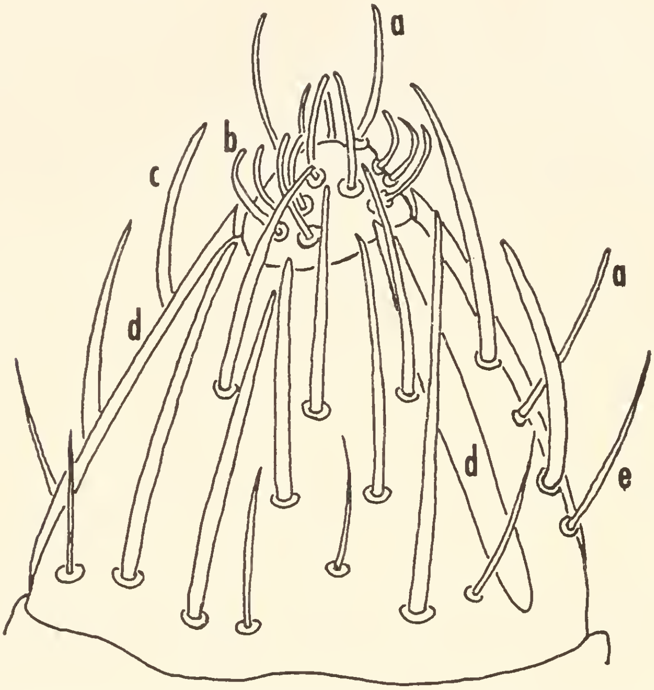

FIGURE 3. Eleventh and twelfth subsegments of male to show all types of sense organs. A, thick-walled chemoreceptor; B, short, curved hair; C, thin-walled chemoreceptor; D, plate organ; E, slender, sharp-tipped hair. Sense organs of subsegment ten that extend forward over the eleventh have been omitted. × 1605.

Chemoreceptors

Three types of chemoreceptors—thick-walled pegs, thin-walled pegs and plate organs with a thin wall—are present on the antennal flagellum of *Nasonia*. The first and second are smaller in size, number and distribution in both sexes while the third occurs in larger numbers in the female than in the male.

I. Thick-walled pegs

These are found only on the eleventh and twelfth subsegments (Figs. 2c, 3a) and are the larger of the two kinds listed by Jacobi (1939, Fig. 29) as present on the terminal subsegment. About six are located on the extreme tip of the flagellum and, since they are longer than the curved hairs described in the preceding section, would be the first to come into contact with any surface to which the end of the antenna is touched. Five or six others occur on the eleventh subsegment. They are about 9 μ long, 1 μ wide at the base, slightly curved and, in contrast to the tactile hairs, have a tip that is rounded. When a solution of crystal violet is applied to the external surface of the antenna, stain enters the tip of the thick-walled peg and, with continued exposure, passes downward towards the base. This indicates that a pore is present at the tip where the dendrites are exposed. The sensory cells innervating these receptors apparently lie within the large mass of neurons present in the tenth and eleventh subsegments and cannot be identified separately. In other species of insects, similar thick-walled chemoreceptors are usually provided with four, five or six neurons. Thick-walled pegs have been found in all species in which a search has been made for them. Those on the mouth-parts and tarsi have been studied intensively, especially in Diptera, by Dethier (1955) and by many others. They occur not only on the antenna but on many other parts of the body as well (Slifer, 1955, 1962). They serve both as olfactory organs and as contact chemoreceptors. Their concentration on the antennal tip in *Nasonia* should aid in the exploration of the surface of the dipteran puparium by the female and may assist the male in recognizing the female.

Since thick-walled pegs occur on many parts of the body besides the antennae in other species, intact *Nasonia* were treated with crystal violet to see whether the pegs were present on the mouth-parts and legs. A single thick-walled chemoreceptor was found on the upper surface of each of the tarsal claws, about five on the terminal portion of each maxillary palp and three or four on the tip of each labial palp. Jacobi (1939) suggested that olfactory organs are present on the palps since females with their antennae removed are still able to react to odors, although more slowly than do normal individuals. He searched for chemoreceptors on the maxillary palps but found only what he believed to be tactile hairs. One of those shown in Fig. 32 of his paper has a rounded tip and is clearly a thick-walled peg of the kind described here.

II. Thin-walled chemoreceptors

A. Thin-walled pegs These, like the thick-walled pegs, have been found on the antennal flagellum of every insect where a thorough search has been made for them. They are transparent, or nearly so, and have a wall that approaches 0.2 μ in thickness and is penetrated by many small openings. Usually they are innervated by a group of neurons although some have been reported with only a single neuron (Boeckh, Kaissling and Schneider, 1960; Dethier, Larsen and Adams, 1963). Each dendrite branches within the peg lumen and delicate pore filaments extend, usually in clusters, from them to the pores in the wall. Here the distal tips of the filaments are exposed to the air.

Thin-walled pegs are present on all subsegments from three to eleven in both males and females. None occur on the first two or on the twelfth. They are about 25 μ long and from 2 to 2.5 μ wide at the base (Figs. 2d, 3c). Each peg is curved and lies close to the antennal wall so that its long axis nearly parallels that of the antenna itself. Jacobi (1939, Figs. 25, 26, 27) gives cross and longitudinal sections through them. Except for a short region at the base, the peg wall is perforated by many small openings. These pores are large enough to be seen easily in sections stained with silver or in whole mounts examined in glycerol (Fig. 4b).

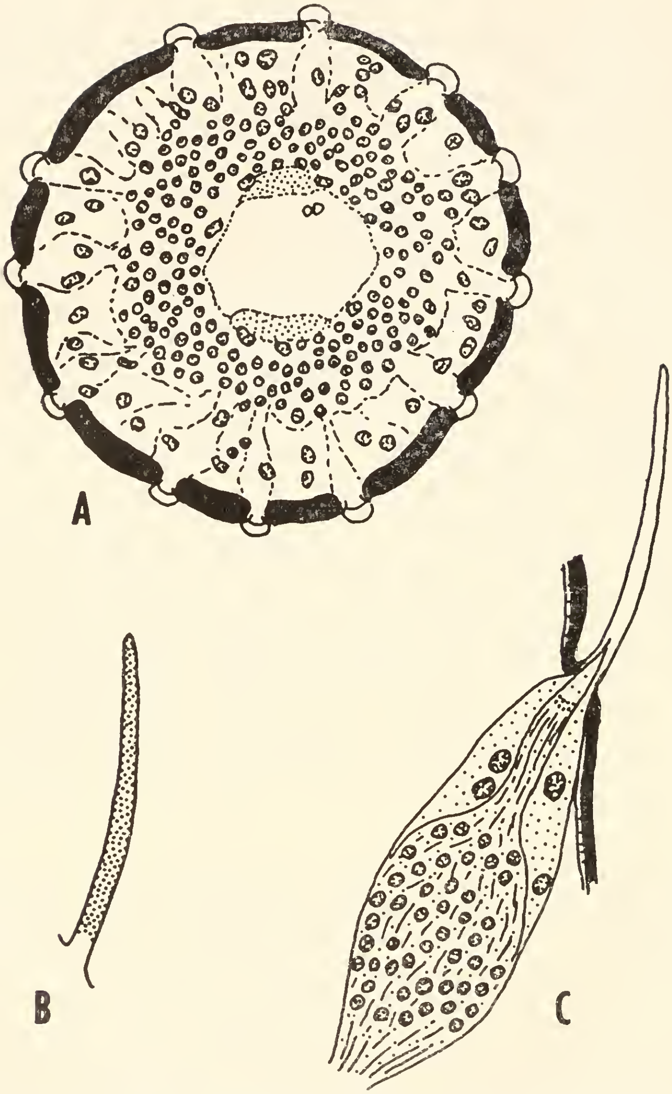

Figure 4. A, cross section of flagellum of female as reconstructed from several sections. Greater part of section occupied by a ring-shaped mass of sensory neuron cell bodies with small nuclei. Large nuclei are those of sheath cells. Central lumen contains two branches of antennal nerve embedded in mass of neurons. Two small circles represent tracheae. Antennal cuticle at periphery includes sections of twelve plate organs. Bouin's fixative, Holmes' silver stain. × 1163. B, whole mount of thin-walled peg from antenna of female examined in glycerol. Pores in wall appear as dark spots on a pale background. Note absence of pores at base. 5% formalin, no stain. × 1778. C, longitudinal section through a thin-walled chemoreceptor from antenna of male. Dendrites from mass of neurons (small nuclei) can be traced to base of peg. Basal bodies (small granules) present on dendrites. Larger nuclei are those of sheath cells. Bouin's fixative, Holmes' silver stain. × 1778.

Stain applied to the outside surface enters the peg readily through them. The thin-walled peg is innervated by a large group of neurons that may lie directly below it (Fig. 4c) or be merged with the larger mass of neurons from many receptors that occupies much of the lumen of the antenna. Several sheath cells (trichogen or tormogen) enclose the dendrites as they approach the base of the peg. Jacobi (1939) shows a group of basal bodies—formerly known as *Riechstäbchen, Sinnesstäbchen* or sense rods—on the dendrites below the peg base and above a large group of neurons. Basal bodies were also seen in the present study in this position (Fig. 4c). We now know, from electron micrographs of antennae of other species of insects, that the sensory dendrite narrows and assumes a ciliary structure immediately above the basal bodies.

B. Plate organs Plate organs in the Hymenoptera have attracted the attention of many workers with the light microscope during the past century but few studies of their fine structure have been made since the electron microscope has been in use. Slifer and Sekhon (1960, 1961) examined the plate organs of the honey bee, *Apis mellifera,* with the electron microscope but staining methods for sections were not available at the time and the work should be repeated. References to earlier literature on the plate organs of various species of ants, wasps and bees may be found in the paper published in 1961.

The plate organ of the honey bee consists of a thick, transparent, oval plate attached to the adjacent cuticle by a thin narrow membrane with radiating lines of minute pores in it. Beneath this thin membrane, but not beneath the plate itself, lies a mass of slender dendrite branches. These arise from a group of sensory neurons that lie below the plate. Recent physiological studies indicate that the plate organ of the honey bee serves as an olfactory organ (Lacher, 1964; Lacher and Schneider, 1963). If this is true, it is highly probable that delicate pore filaments extend from the dendrites to the pores as they do in the plate organ of the aphid (Slifer, Sekhon and Lees, 1964). The function of the thick, transparent plate that makes up the greater part of the surface of the receptor in the honey bee remains unknown.

In *Nasonia* plate organs are present on all of the subsegments except the first, second and twelfth. Jacobi (1939) counted the plate organs on subsegments three to eleven for a single antenna of a female. The results, in order, were 6, 5, 6, 6, 9, 9, 14, 14 and 5 with a total of 74. In the present study the plates on six antennae from males and six from females were counted. The results are shown in Table I. It will be noted that, with a few exceptions, the number of receptors rises for successive subsegments and then falls sharply at the eleventh. The mean total for males is 43 and for females 81. According to Cousin (1933), the removal of the antennae from a female suppresses reflexes that make mating possible but this is not the case for the male. It would be interesting to know whether the difference in the number of plate organs is in any way concerned. Perhaps they aid the female in finding decomposing material in which dipteran pupae may be found although it should be noted that Cousin (1933) states that females without antennae are still able to find such pupae.

The plate organs of *Nasonia* are the largest and most conspicuous of the antennal receptors (Figs. 2e, 3d). They are transparent, elongated structures that are raised above the antennal surface and arranged, more or less regularly, around the subsegments (Figs. 3, 4a). In life they stand out conspicuously against the dark antennal cuticle. The mean length of those measured on an antenna from a female was 31 μ and the same figure was obtained for the antenna of a male. Their maximum width is about 5 μ. When the insect is immersed in a solution of crystal violet, the stain enters rapidly through the plate organs. This provides evidence that the plate has pores in it and that the structures serve as chemoreceptors. In sections cut in a plane parallel to the surface of the antenna and stained with Holmes' silver technique the porous nature of the plate is definitely confirmed (Fig. 5a). The pores are smaller than are those of the thin-walled pegs and careful focusing of the microscope is necessary before they can be seen clearly. The entire outer surface is uniformly covered with the small pores. The relative ease with which pores can be demonstrated in *Nasonia* contrasts strongly with the situation in the honey bee plate organ where it has not yet been possible to demonstrate pores with the light microscope.

Table I

Number of plate organs on flagellar subsegments of antennae of males and females

Subsegment	3	4	5	6	7	8	9	10	11	Total
♂♂	3	2	4	5	5	4	6	5	2	36
	5	4	5	6	4	4	6	7	3	44
	4	4	4	5	5	7	6	6	3	44
	3	3	5	5	6	6	7	6	3	44
	2	3	3	4	7	5	7	6	3	40
	5	4	4	6	7	8	7	7	4	52
									Mean total	43
♀♀	4	5	5	8	9	11	10	13	4	69
	3	5	5	7	11	11	11	13	3	69
	7	6	8	7	10	10	15	15	6	84
	5	5	8	9	12	13	14	13	5	84
	6	7	8	8	12	12	15	14	6	88
	5	7	7	8	12	14	15	15	6	89
									Mean total	81

In cross sections of the antenna a thin membrane can be seen extending across the receptor about 1.5 μ below the outer surface (Figs. 4a, 5d). This lies just above two shelf-like invaginations of the cuticle that may help to support it. Near the proximal end of the plate a large group of dendrites extends upward towards the membrane (Figs. 5c, 5d). Presumably these pass through one or more openings in the inner membrane as they do in the aphid plate organ (Slifer, Sekhon and Lees, 1964), then enter the outer chamber, branch and send filaments into the fine pores in the surface.

Basal bodies are present in the dendrites and may be seen as a band of dark spots 4 or 5 μ below the surface of the receptor (Fig. 5d). Jacobi (1939) evidently did not see them for there are none in his longitudinal section of a plate organ. The dendrites of the sensory neurons of the plate organs, as well as those

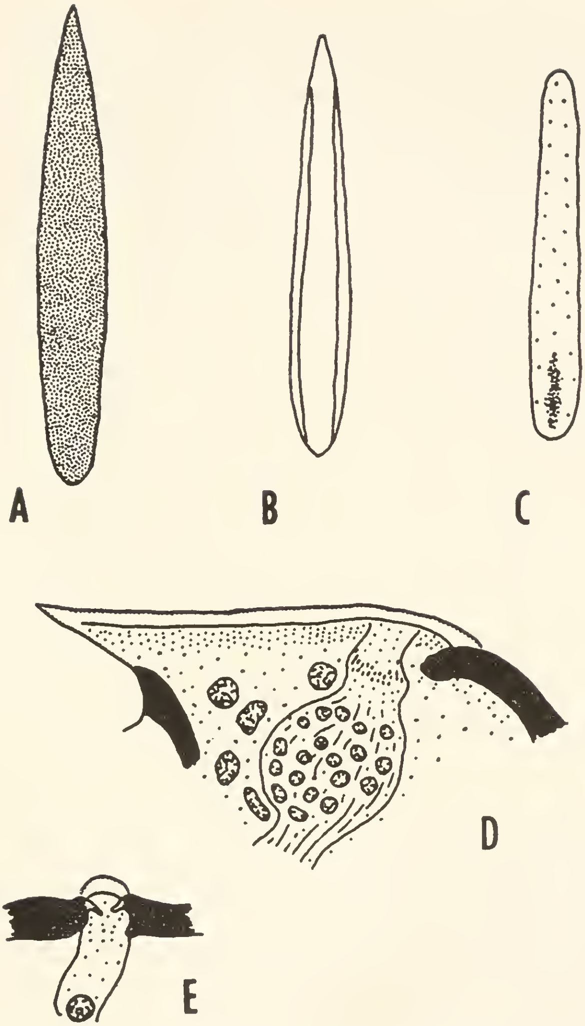

FIGURE 5. A, surface view of plate organ showing fine pores in wall. Compare with D and E. B, view through plate organ at lower focal level than that shown in A. Note two shelf-like cuticular extensions along sides. Compare with E. C, view through plate organ at still lower level and showing cluster of basal bodies at proximal end. Compare with D. D, longitudinal section of plate organ showing surface with fine pores and delicate inner membrane that separates outer chamber from parts below. The mass of neurons (small nuclei) sends dendrites toward the outer chamber. Basal bodies (small granules) present on dendrites. Larger nuclei are those of sheath cells. Compare with A, B, C and E. E, cross section through outer part of plate organ to show relations of surface with pores, delicate membrane that forms floor of outer chamber and shelf-like cuticular extensions just below it. Dendrites not included in this section. Nucleus is that of a sheath cell. All Bouin's fixative, Holmes' silver stain. × 1850.

of other receptors on the antenna of *Nasonia,* are not enclosed within a tubular cuticular sheath as they are in many insect species. This was also noted for the sense organs of *Apis mellifera* investigated earlier (Slifer and Sekhon, 1961). The group of neurons that innervates the plate organ is large and may lie directly below the plate or, more often, form a part of the compact and massive ring of neuron cell bodies that occupies much of the antennal lumen (Fig. 4a). The sheath cells that are responsible for the secretion of the cuticular parts of the plate organs encircle the dendrites and fill the region below the inner membrane. Their nuclei are larger than are those of the neurons and their free borders are covered with microvilli. The epidermal cells that lie below the antennal cuticle elsewhere are much flattened and difficult to see in sections although their nuclei can be identified.

The arrangement of the mass of neurons within the lumen of the antenna is of some interest. A single large antennal nerve traverses the scape, pedicel and first two flagellar subsegments. In the second subsegment a small mass of neuron cell bodies lies at one side of the nerve. In the third subsegment the nerve branches into two and these, decreasing in size, can be traced into the tenth subsegment. Within each subsegment from the third to the eighth the nerves are surrounded by and embedded in a large ring-shaped mass of neuron cell bodies (Fig. 4a). The masses are larger in the females since in this sex there are nearly twice as many plate organs as there are in the males. Between subsegments, where the antenna is strongly constricted, the two antennal nerves are the only sensory elements present. The ninth and tenth subsegment each contains a large mass of neurons but those of the eleventh, combined with those of the twelfth, lie in the distal end of the tenth and proximal ends of the eleventh subsegments.

SUMMARY

1. Although the male of *Nasonia vitripennis* is distinctly smaller than the female, the antennae are approximately the same size in both sexes.

2. Hairs of two kinds, both believed to be tactile, are present on the antennae of both males and females. One type is found only on the twelfth subsegment.

3. Chemoreceptors of three kinds—thick-walled pegs, thin-walled pegs and plate organs—occur on the antennal flagellum of both sexes.

4. Thick-walled pegs are restricted to the eleventh and twelfth subsegments of the antenna. Elsewhere on the body they were found on the terminal segments of the maxillary and labial palps and on the tarsal claws.

5. Thin-walled pegs are present in large numbers on all subsegments from the third to the eleventh.

6. Plate organs occur on all subsegments from the third to the eleventh. A mean number of 43 plate organs was found on the antenna of the male and 81 on the antenna of the female. The entire outer surface of the plate is perforated by a large number of very small openings.

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PHOTOPERIOD CONTROL OF DIAPAUSE IN *DAPHNIA*. II. INDUCTION OF WINTER DIAPAUSE IN THE ARCTIC

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The sexual polymorphism in *Daphnia* which results in an embryonic diapause is facultative, and its onset may be under the control of photoperiod and a density proportional stimulus (Stross and Hill, 1965, 1968). In the previous study a population of *D. pulex* which overwinters in an embryonic diapause at temperate latitude (45° N) responded to culture density when the photoperiod was 13 hours of light per day. At an appropriate density a photoperiod response curve, typical of other arthropods, was generated. The critical photoperiod was also temperature compensated in the range tested, 12° and 19° C. This report describes an extension of that study to arctic latitudes where an effort was made to determine if the reproductive cycle of arctic *Daphnia* may be under photoperiod control.

Facultative diapause is not readily apparent in arctic populations of arthropods from descriptions of life history. The brief summer season may allow only a single generation of insects to develop. At Barrow, Alaska (71°) a fresh-water calanoid copepod (Comita, 1956) and the Cladoceran, *Daphnia middendorffiana* Fischer (Edmondson, 1955) are reported to develop essentially only one generation each year. Univoltinism is not necessarily obligatory in arthropods at high latitudes as has been demonstrated (Danilevskii, 1965; Morris, 1967). Indeed the reproductive cycle of *D. middendorffiana* in a lake includes one or two broods of non-diapausing embryos before the population shifts. Populations of the same species at Cape Thompson, Alaska (68° N), where the ice-free season is longer are reported to shift at approximately the same time in the late autumn of the arctic (Hilliard and Tash, 1966).

The species *D. middendorffiana* is apparently widely distributed in the myriad small pools characteristic of the frost-patterned earth of the arctic. Brooks (1957) describes it as phylogenetically distinct from *D. pulex* which he excludes from the arctic. The only other species reported by him to occur in arctic Alaska near Barrow is *D. longiremis* a much smaller species which apparently overwinters in a non-embryonic stage. The second species is characteristic of lakes, and, with the exception of Imikpuk Lake which contains *D. middendorffiana* and fairy shrimp but no fish or other dangerous predators, it may be found in fish-containing lakes such as Ikroavik (Wohlschlag, 1957) and Sungoroak near Barrow.

METHODS AND MATERIALS

Daylength, culture density and temperature were tested as potential stimuli controlling the reproductive shift of *Daphnia middendorffiana* Fischer. The laboratory experiments were complemented by simultaneous observation of the reproductive cycle in the arctic pool from which the experimental stock was removed.

The study began on June 15 at Barrow when overwintering embryos, still in their egg pods (ephippia) were transferred to the laboratory. The embryos hatching in continuous light were used in the first experiments and to provide an uncloned stock for measurement of response in the first and second generations.

Culture techniques were as described previously (Stross and Hill, 1968). They consist of rearing young, 0 to 2 days of age for a period of usually 30 days at controlled densities. At two-day intervals the test animals are transferred to fresh suspensions of food, and released broods are censused. Water from the source pool served as culture medium after it had been filtered through a plankton net (mesh opening, 50 microns). A synthetic medium, previously described was used as a control. The food medium consisted of a mixture of axenically grown cells of *Chlamydomonas reinhardti* (Indiana U. strain #90) and a nearly unialgal suspension of *Kirchneriella* (*subsolitaria* ?) which was grown in a greenhouse aquarium under natural daylight; the mixture was used with some indication that *Chlamydomonas* alone might be unsatisfactory. Initial cell densities ranged from 0.9 to 1.2 $\times 10^6$ cells/ml in the cultures during the course of the investigation.

The *Daphnia* cultures were housed in water baths at 12.5 or $20.0 \pm 1.0°$ C. Illumination, controlled by electric timers, was supplied as fluorescent (cool white) light at intensities of 125 and 28 ft-c at the level of the cultures.

Field observations consisted of weekly collections of duplicate samples from the source pool (Near Ditch) and in semi-weekly census of small (400 ml) transparent enclosures suspended in the source pool. Dimensions of the pool in June were approximately 3 meters $\times 1\frac{1}{2}$ meters $\times \frac{1}{2}$ meter deep (maximum). The volume later shrank as a result of evaporation and withdrawal of medium for the laboratory experiments. Temperatures of the pool were recorded with a Taylor max-min thermometer and Foxboro recording thermometer. Details of water chemistry, algal populations, *etc.* have been described for the arctic pools near Barrow (Kalff, 1967).

The reproductive cycle of *Daphnia* was examined in three other small pools near the Arctic Research Laboratory and in Imikpuk, Ikroavik and Sungoroak lakes near Barrow, Alaska. A single observation was made on the status of the reproductive cycle in one of the lakes at Cape Thompson (68° N) previously studied by Hilliard and Tash (1966).

Results

The reproductive shift which leads to the diapausing embryo may be determined by daylength. Photoperiod control is expressed more clearly by the generations born to the overwintering generation. All broods are (ephippial) diapausing at photoperiods of 20 hours (L:D 20:4) or shorter including only 15 minutes of light per day (Fig. 1). The incidence or fraction of diapausing broods at daylengths longer than 20 hours is influenced by density of culture. At the minimum density of one per container (20 ml), no broods (5 replicates) are diapausing in constant light. Densities of 3 or 5/20 ml result in 20 or 50 per cent, respectively, diapausing broods in constant light. A critical photoperiod (ratio of 1:1 diapausing to non-diapausing broods for the test period) is approximately L:D 22:2 as shown by the response at densities of 1 and 3/20 ml. The densest cultures (5/20 ml) resulted in an apparent shift of the critical photoperiod to a longer daylength (Fig. 1).

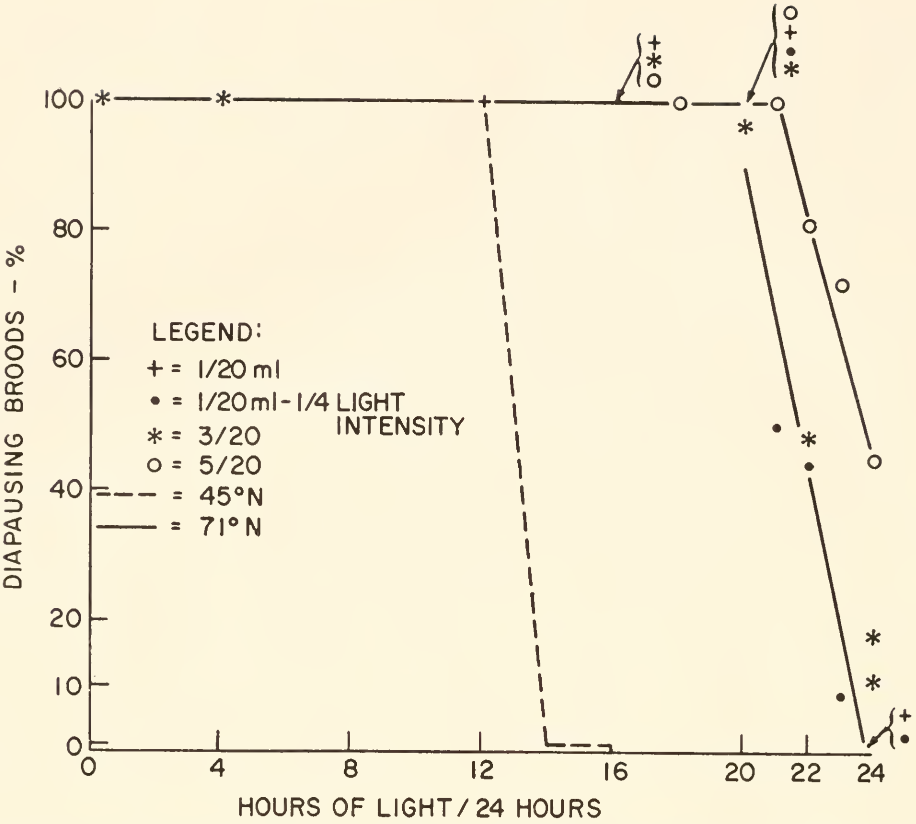

FIGURE 1. Reproductive shift of *Daphnia middendorffiana* in response to daylength and culture density. First and second-generation young born in constant light and transferred to photoperiod indicated. Photoperiod response of winter diapausing population of *D. pulex* at 45° N is shown for comparison. All experiments at 12° C.

Starvation may be discounted as a stimulus associated with culture density. All densities received the same bi-daily ration of food, and this is reflected in the number of young in broods of non-diapausing embryos. At 12° mean brood size for the test interval, which includes the first four or five broods of the adult reproductive period was 13.3 young brood at a density of 1/20 ml and 5.5 and 3.2 young at densities of 3 and 5/20 ml, respectively, in constant light.

Overwintering generation

Daylength and density may also control the reproductive shift in the overwintering generation. These stimuli are effective, however, only after the adults have produced one or two broods of non-diapausing embryos (or young) and under conditions which are inductive for first and second generation adults. Young which had spent most of their prenatal development in constant light, either in the field or in the laboratory were subjected to four photoperiods at densities of

3, 5 and 10/20 ml. The food ration was doubled for the densest cultures. The incidence of diapause increased with decreasing daylength and with increasing density. Both variables were significant ($P = 0.01$) in a two-way analysis of variance (5 replicates) at both 12 and 20° C. Although significant, the variables clearly fail to generate the elegant all-or-none response provided when generations of the year were tested.

The number of non-diapause broods produced by the overwintering generation before the reproductive shift is variable and may be determined by photoperiod and culture density. At 12° and constant light the first two broods produced by each female at minimum density (3/20 ml) were virtually all (26 of 27) non-diapausing (Fig. 2). Denser cultures produced fewer non-diapausing broods such that at a density of 10 adults/20 ml most of the females released only one brood of young before switching. At inductive photoperiods the first brood was non-diapausing in all but the densest cultures. Broods subsequent to the first were nearly all diapausing at inductively short photoperiods. In constant light, the only non-inductive photoperiod tested, some of the third or later broods were non-diapausing even in the densest cultures. The response to photoperiod and density was similar at 19° that is, the temperature effect in a three-way ANOV was not significant.

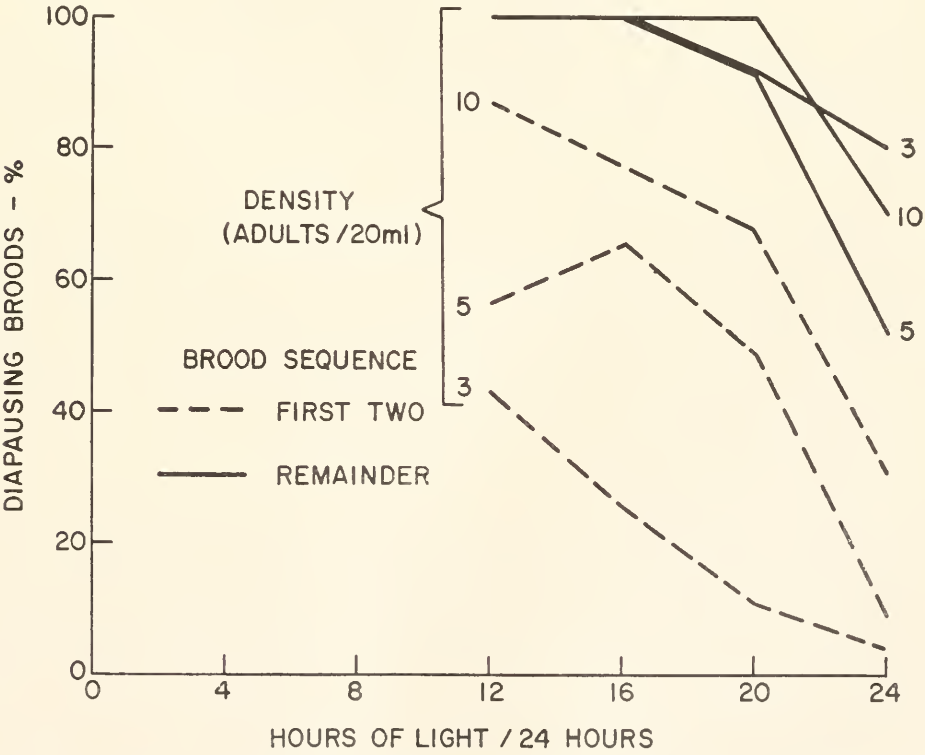

FIGURE 2. Reproductive shift of overwintering generation of *D. middendorffiana* in response to photoperiod and density at a temperature of 12° C.

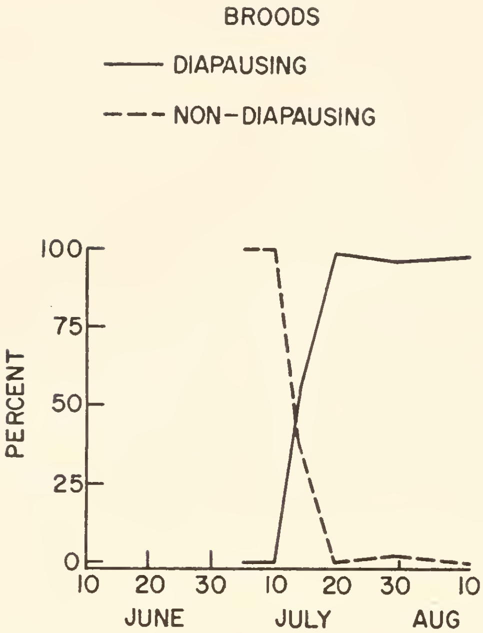

FIGURE 3. Reproductive shift of *D. middendorffiana* in the arctic pool supplying laboratory stock, as determined by inspection of brood contents of the adults. The sun is continuously above the horizon until August 3.

Males

Males are reported to be non-functional in *D. middendorffiana* (Edmondson, 1955; Brooks, 1957), as determined from examination of the testes. These conclusions are supported in this study by the successful rearing of the diapaused embryos developed by females isolated since birth. Although rare in nature, males are produced. In the source pool, the mid-August collection contained 4.8 per cent males. Hosseinie (1966) reports having obtained them regularly in cultures. In this study a single male was discovered to have been produced in culture, of some 1500 first and second generation individuals used in the experiments.

Reproductive cycle in the field

In the laboratory the reproductive switch may be judged facultative and under the control of photoperiod. The critical photoperiod of L:D 22:2 would permit the reproductive shift to begin on or about September 1, depending on the requisite number of inductive light-dark cycles required by the adults. A shift at that time is consistent with the major shift reported by Edmondson (1955) for Imikpuk Lake at Barrow and by Hilliard and Tash (1966) for small lakes at Cape Thompson, Alaska (68° N).

In the pool environment the reproductive shift is in mid-July, two weeks before the first sunset of the summer and six weeks earlier than a direct extension of laboratory results would permit. The mid-July shift was recorded for the source pool (Fig. 3) and in two other nearby pools. A fourth pool deepened by previous passage of tracked vehicles shifted in late July. In the source pool the population apparently produced only one brood of non-diapausing embryos before shifting or 2.65 young per adult, as measured in the exclusion containers. They were released during the interval of July 10 and 17. During the following month, or from July 17 to August 15, each adult produced 2.3 diapausing embryos (1.4 egg pods) most of them during the first two weeks after the shift.

On a seasonal basis the synchrony in population growth and development is highly compressed. In 1967 overwintering embryos hatched on June 19 and 20 one week after the pool was free of ice and on the same days that embryos transferred to the laboratory hatched when held at 11.5°. Two weeks later oviposition into the brood pouch began; 7 per cent of the population was gravid on July 5, and 48 per cent were carrying embryos two days later. Essentially all of the young were born between July 10 and 17. The reproductive shift was equally compressed. On July 10 all gravid females were carrying non-diapausing embryos (Fig. 3). Four days later 55 per cent of the gravid females were developing broods of diapausing embryos; 80 per cent of the adults were gravid at this time. On July 17, egg pods (ephippia) containing the diapausing embryos were being released and 95 per cent of the developing broods were diapausing. The density of Daphnia on July 10 was estimated at approximately 60/liter.

Development is more rapid in the pools, than in Imikpuk Lake (Edmondson, 1955). Temperatures in the pools averaged 10° or 11° from mid-June until the end of July. The daily oscillation ranged from 4° to 7° at approximately 0300 to 12° to 15° C at 1500 hours; the oscillation may be damped near either extreme by the time of day when clouds or fog blanket the earth. In Imikpuk the temperature lags such that a maximum temperature of 10° is not attained until late July following the disappearance of ice on July 19 (Edmondson, 1955).

The timing of the reproductive shift in Imikpuk is confirmed in this study. Gravid female *Daphnia* collected on August 15 contained only broods of non-diapausing embryos. On August 29 the population was in transition since 30 per cent of the gravid females were carrying broods of diapausing embryos. Gravid females in the population in a lake at Cape Thompson contained 38 per cent diapausing broods when sampled three days earlier.

Reversal of reproductive shift

Reproduction may revert to non-diapause when the females are placed in the appropriate environment. In the first experiment females from overwintering embryos were diluted from a density of 10/20 ml to a density of 3/20 ml after they had released the first brood. In constant light the incidence of diapausing broods was reduced to 43.5 per cent as compared with 92.0 per cent in undiluted controls. There was no reversal following dilution at L:D 20:4. In the second experiment adults were transferred from the source pool on August 1 where they had been under continuous light. Cultured at a density of 1/20 ml in constant light, the females reverted to an extent predicted by the photoperiod response curve (Fig. 1). In constant light 3 of 4 reverted to the production of non-diapausing embryos. At L:D 20:4 and 21:3 none reversed, while at 22:2, 2 of 4 reverted. The response was the same at both light intensities.

The response of the field-collected animals differed in the time required for reversal to occur. The laboratory crowded animals (Expt. 1) reverted quickly and there was no difference in incidence of diapausing broods in the first and second 15-day intervals following dilution. Animals reared in the pools required 22 or 26 days to release the first non-diapausing broods. Both experiments were carried out at 12° C.

Synchrony in laboratory and field

Several lines of evidence indicate a moderate degree of synchrony in field populations in the presence of continuous light. Embryos transferred to the laboratory at constant temperatures hatched synchronously. At 12° 95 per cent (271) of the total hatched in 24 hours. At 20° the same percentage (231 young) hatched within 12 hours. Molting and release of brood-pouch contents among five replicates of single individuals, brought in as young from the source pool, remained in step, at least within the two-day interval between censuses (Fig. 4).

Instar duration and reproductive polymorphism

The synchrony of molting and release of broods of young or diapaused embryos (Fig. 4) permits an analysis of instar duration. The interval between successive broods of young was 4.7 days at 13° C. The interval between the release of an egg pod (ephippium) was 6.0 days at 13° C. However, the actual duration of a maternal instar which produces a brood of diapausing embryos is only one-half of the 6 days or 3 days. A barren instar intervenes between instars producing diapausing broods (not shown in Fig. 4) such that the instar is roughly two-thirds the length of an instar producing a brood of young. The intervening barren instar, apparent from the shed exuvium was first pointed out by Leary (1967) who reported barren instars for a population of *D. pulex* (Paul Lake, Mich.). Relative duration of instars was reported to be the same at 11° C. At 20° C diapausing instars were only 76 per cent as long. The longer duration required for release of a brood of diapausing embryos and the lack of fractional days in the average interval between release suggest the ovarian cycle is more rigidly entrained to a phase of the light-dark cycle.

Discussion and Conclusions

The reproductive polymorphism in an arctic (71° N) population of *Daphnia middendorffina* is facultative, and the expression which leads to the development of diapausing embryos is under the control of environment. In the laboratory under constant temperature and standardized densities, photoperiod controls the reproductive shift. A critical photoperiod of L:D 22:2 is the longest known and nine hours longer than the critical photoperiod of 13:11 for a population of *Daphnia pulex* at 45° N (Stross and Hill, 1968). In responding the arctic population provides additional evidence for photoperiod involvement in the reproductive shift. Since the most dilute culture of one individual in 20 ml gave a complete response, there is no proof that a crowding stimulus is required in conjunction with a permissively short photoperiod. Nevertheless, the much longer critical photoperiod is evidence for latitudinal adjustment shown for other arthropods at mid and high

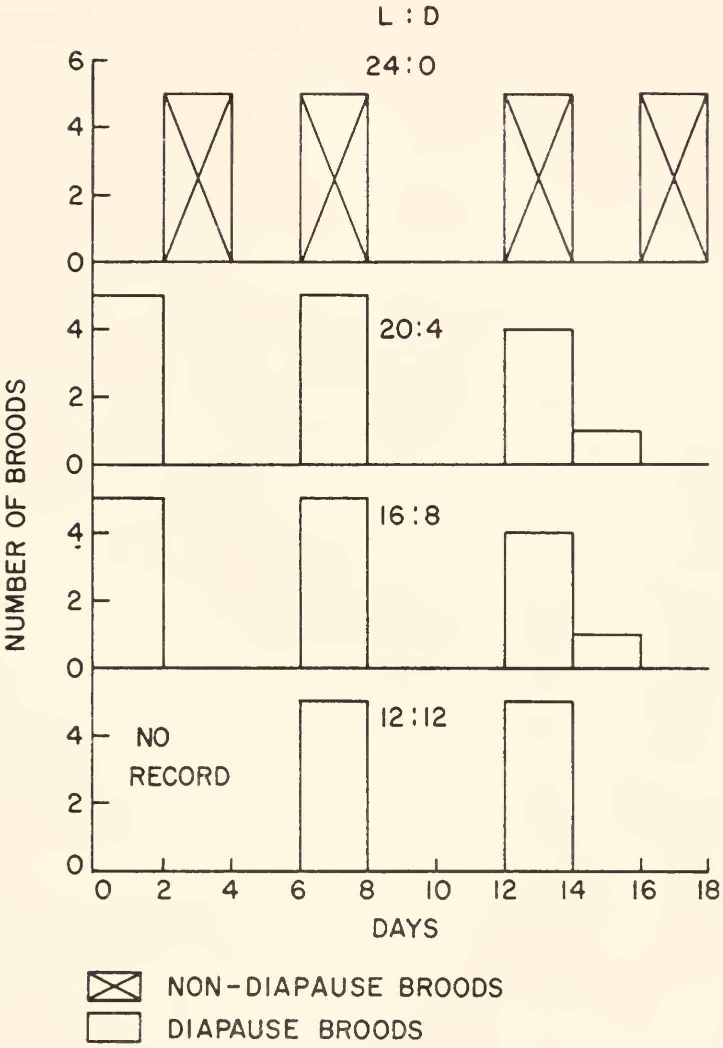

FIGURE 4. Synchrony of brood release in five replicates of single individuals per container at photoperiods indicated. Cultures examined at bi-daily intervals. Results plotted as the number of broods released during a two-day interval between inspection. *Daphnia middendorffiana* collected as first generation young from source pool and cultured at 12° C.

latitudes (Danilevskii, 1965), but not at low latitudes (Ankersmit and Adkisson, 1967) and required by Hutchinson (1967) for a general acceptance of photoperiod control of the reproductive polymorphism in Cladocera.

In the field, populations shift in the source pool near Barrow well in advance of prediction, and it might be argued that crowding overrides photoperiod control, as it may in the laboratory. Several facts argue against this, however. The shift may be avoided in the laboratory cultures containing water from the same pool. The density of *Daphnia* in the pool was only slightly greater than 1 adult/20 ml which gave no diapausing broods in constant light and much less than 3/20 which gave only partial overriding in constant light. Indeed a density of 10/20 ml permitted some non-diapausing broods. Yet in the field the shift was virtually complete.

Starvation is another possibility since a food supplement was added to the cultures. At the time of the shift in the pool, 80 per cent of the adults were gravid and that level of nutrition argues strongly against starvation.

The most likely candidate interacting with photoperiod to cause a reproductive shift is temperature. Either the low temperature or the large thermal oscillation may interfere with a photoperiod effected process as shown for insects (Pittendrigh, 1954; Saunders, 1967, 1968). Admittedly speculative, the last possibility is offered to counteract the inference that density is the most likely possibility by extension of the laboratory result. Thermal interaction with a long-day stimulus has many attractive possibilities. It could permit selection for a long critical photoperiod in arctic pools which, rather than the lakes, are probably the ancestral and certainly are the present principal location of the species. Thermal interference could explain the dramatic shift from all non-diapausing to virtually all diapausing broods in the pools. The slow reversal of *Daphnia* reared in the field suggests actual reversal of short-day induction and contrasts with the rapidity with which laboratory cultures responded when the suppressive effect of crowding was withdrawn in a long-day environment. In other words the populations in the pools may behave as if they spend the entire active phase of the life cycle in a short-day environment despite the continuous light. The resistance of the overwintering generation of *Daphnia* to short-day photoperiods is analogous to the response of females from overwintering embryos (fundatrix) in aphids (Lees, 1966) another seasonally polymorphic group. In *Daphnia* the resistance is restricted apparently to permitting birth of only one brood of young each year. The significance of population expansion each year and proof for the temperature postulate require further analysis.

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SUMMARY

1. Control of the sexual polymorphism leading to an embryonic diapause was studied in an arctic (71° N) population of *Daphnia middendorffiana*. The reproductive cycle is similar to a previously analyzed population of *D. pulex* although males are non-functional in the polymorphism.

2. In constant temperatures and standardized culture density the reproductive shift is controlled by daylength with a critical photoperiod of L:D 22:2 at 12° C. Control may be partially overridden in long days (constant light) by density of culture. The overwintering generation shows resistance to both photoperiodic

induction and density suppression, and one or two non-diapause broods are released before reproduction shifts.

3. The reproductive shift in the source pool at Barrow, Alaska occurs in mid-July when the sun is continuously above the horizon and after the overwintering generation has released one brood of non-diapausing embryos. Completeness of the shift and the relatively low density of the population in the pool argues against a density override of photoperiod control. Another component of the environment which may create effectively short daylengths in the presence of continuous light is postulated. The large oscillation in temperature or low minimum which occur daily could be involved. The reproductive shift in a lake at Barrow and at Cape Thompson (68° N) is consistent with photoperiod control.

4. Results of this study support the previous hypothesis in part, namely, that the sexual polymorphism in short-day induced populations of *Daphnia* may be under the control of photoperiod. Also shown is the latitudinal adjustment necessary for functioning of photoperiod at mid and high latitudes.

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RESPIRATORY ADAPTATIONS OF TWO BURROWING CRUSTACEANS, *CALLIANASSA CALIFORNIENSIS* AND *UPOGEBIA PUGETTENSIS* (DECAPODA, THALASSINIDEA) [1]

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Patterns of metabolic regulation among Crustacea range from metabolic dependence upon external oxygen tension to metabolic independence (Prosser and Brown, 1961; Wolvekamp and Waterman, 1960). Patterns of regulation are generally related to the ecology of the species concerned but only rarely are direct correlations made with the availability of oxygen in the habitat.

Thalassinid mud shrimps have a long evolutionary history as burrowers in marine sediments (Borradaile, 1903). It is well substantiated that mudflats are primarily hypoxic environments and presumably oxygen availability is a limiting factor to mudflat inhabitants (Brafield, 1964; Krogh, 1941; Pearse, Humm and Wharton, 1942). It may be expected that success in these habitats is in part predicated upon metabolic adaptation.

Very few published accounts of thalassinid respiration exist (Montuori, 1913). The purpose of the present investigation was to determine the effect of oxygen tension on the metabolic rate of two shrimps, *Callianassa californiensis* Dana and *Upogebia pugettensis* (Dana). Tolerance of anoxia was studied and preliminary measurements of post-anoxic respiration were made. This report attempts to relate ecological observations to the respiratory physiology of each species.

MATERIALS AND METHODS

This study was conducted at the Oregon State University Marine Science Laboratory, Newport, Oregon, from April through June, 1966. Shrimps were collected at low tides from the exposed mudflats of Yaquina Bay. Specimens of *Upogebia pugettensis* were dug near Coquille Point on the north shore of the bay. A "shrimp gun," a cylindrical suction device similar to a "yabby pump" (Hailstone and Stephenson, 1961) was used to obtain *Callianassa californiensis* from the south shore of the bay near the marine laboratory.

Immediately after collection the shrimp were taken to the laboratory. The number collected, size, sex, and reproductive state were recorded at this time. The stages of the molt cycle could not be accurately determined; however postmolt individuals could be singled out by their light color, white setae, and softness of exoskeleton. All shrimp were maintained in containers previously filled with sand or mud obtained from the respective collecting areas, and were provided with flowing sea water (SW). The unfiltered SW contained sufficient organic detritus to maintain a number of animals in apparently good condition for as long as three months, as judged by full hind-guts and the formation of faecal pellets. No attempts were made to starve the animals. None of the animals used for experimental purposes was ever under laboratory conditions for more than two weeks.

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The salinity of the laboratory SW ranged from 25.6‰ to 34.6‰ during the study period. The temperature range in the laboratory tanks during April–May was from 9°–13° C, but was slightly higher in June (10°–15° C).

Metabolic rate-oxygen tension measurements

An oxygen macro-electrode was used in conjunction with a Beckman Physiological Gas Analyzer (model 160) to determine the oxygen tension of SW under laboratory conditions. The oxygen electrode and the analyzer were calibrated before each experiment. Calibration solutions were made with SW whose salinity was identical to that used throughout each experiment. Since electrode performance varies widely with temperature, calibration was made at the temperature at which the sample measurements were to be made. All experiments and calibrations were carried out in a water bath at 10° C ± 0.2° C.

A zero oxygen calibration solution was obtained by using "Oxsorbent" (Burrell Corp.). Compressed air was used to obtain a fully air-saturated calibration solution. A value of 20.95% was used to express the concentration of oxygen in air. Empirical calibration of the gas analyzer meter was necessary to yield an absolute scale. Hence, SW samples representing different saturations were analyzed for oxygen content by a modified micro-Winkler method. In this fashion oxygen tension (mm Hg) was found to be directly proportional to oxygen concentration (ml/l) over the full range from zero to 100% air-saturation.

The following conditions were observed in all experiments. Only adult male shrimp, ranging from 3.4–8.7 g and in apparently good condition, were used. Animals were removed directly from the sand or mud substrate in the laboratory and placed in either a 0.2-liter or 0.4-liter jar, depending on the size of the shrimp, for one to two hours before the beginning of an experiment. During this period of adjustment the SW was kept at or near full air-saturation by aeration. The salinity of the water ranged from 31–35‰.

Within the jar the animal rested on a plastic screen supported above a magnetic stirring bar by a lucite cylinder. A water-driven underwater stirrer prevented stratification of oxygen within the jar. The performance of the oxygen electrode was empirically determined to be independent of the stirring rate. Rates as low as possible were used to minimize disturbance to the animal. Although activity of individuals was not monitored, animals were for the most part quiescent during determinations.

At the beginning of an experiment the jar was sealed with a rubber stopper through which the previously calibrated electrode and a breeder line had been inserted. As the shrimp depleted the oxygen the drop in oxygen tension was recorded from the analyzer at 15-minute or one-half-hour intervals. To arrive at an accurate measure of the volume of the jar, the volume of the animals was determined by water displacement. Wet weights of the shrimp were taken on a Mettler balance at the completion of the run. All experiments were started around noon, lasting until the individual shrimp had lowered the oxygen tension to zero, *ca.* 12–24 hours. Using the micro-Winkler calibration values and the jar volume, the resulting time-tension curves were then translated into oxygen consumed per given time interval; final calculation resulted in ml O_2 consumed × gm wet body weight^{-1} × hr^{-1}. For comparative purposes 100% air-saturated SW at 10° C is equivalent to an oxygen tension of 160 mm Hg or a concentration of 6 ml O_2/l.

Heart rate measurements

The effect of slowly decreasing oxygen tension on the heart rate of *C. californiensis* was measured. The heart is covered by a relatively transparent carapace; hence, the heart rate can be easily counted in the intact animal. A rectangular lucite box was constructed to permit regulation of the oxygen tension of the SW flowing over the shrimp. To minimize movement the shrimp was confined within the box by a glass tube whose open ends were covered with pieces of plastic screen. SW was siphoned through this chamber at rate of *ca.* 25 ml/min. The entire system was maintained at 10° C. The experimental animal was placed in the chamber one to two hours before counting commenced. The time for ten heartbeats was measured with the aid of a dissecting microscope. After heart rates were obtained at air-saturation, the oxygen tension was lowered slowly by bubbling nitrogen gas through the SW reservoir. Periodically a micro-Winkler sample was taken from the box and the heart rate was measured immediately thereafter. This procedure was repeated until anoxic conditions were obtained.

Survival time under anoxic conditions

Specimens of *C. californiensis* and *U. pugettensis* were placed in deoxygenated water and maintained until death occurred. Anoxic conditions were obtained by bubbling nitrogen gas through full-strength SW for 1–2 hours. At the end of this time, when shrimp were introduced, only a very small amount of oxygen remained in the water (average 0.011 ml/l). This was considered negligible and was consumed during the experiment, since a micro-Winkler sample at the termination of the experiment yielded essentially zero oxygen. Except for one group experiment (12 shrimp/3.8-liter jar), the shrimp were individually marked and put into jars of anoxic water, which were then sealed tightly and held at 10° C.

Burrow and interstitial water sampling

Water samples from burrows of *Upogebia* were analyzed for dissolved oxygen by the micro-Winkler procedure. Burrows were randomly selected yet checked for signs of recent substrate activity and for the presence of faecal pellets, both of which are indicators of shrimp habitation. To obtain the water samples soft plastic tubing (5 mm diameter) was carefully threaded into the firm burrows (12–20 mm diameter) to a minimum depth of 30 cm. Sample water (10 ml) was drawn up into a glass syringe in such a fashion that no air bubbles were introduced. Large amounts of sand and/or mud were kept from entering the syringe by covering the end of the sampling tube with cheesecloth. Syringes were sealed with toothpicks, placed on ice and taken directly to the laboratory for analysis.

Water samples from burrows of *Callianassa* were not obtainable because the burrows were collapsible and relatively impermanent. Only interstitial water samples were taken. An interstitial water sampler was constructed of hard, clear, plastic tubing (93 cm long × 1.9 cm diameter) with small holes drilled around the circumference for a distance of 15 cm from the bottom. A solid pointed end enabled the sampler to penetrate 30–60 cm into the substrate. A 10-ml sample of interstitial water was collected and then treated as described above for burrow samples. Sufficient interstitial water samples were not obtainable from the habitats of *Upogebia* because of the exceptionally fine substrate.

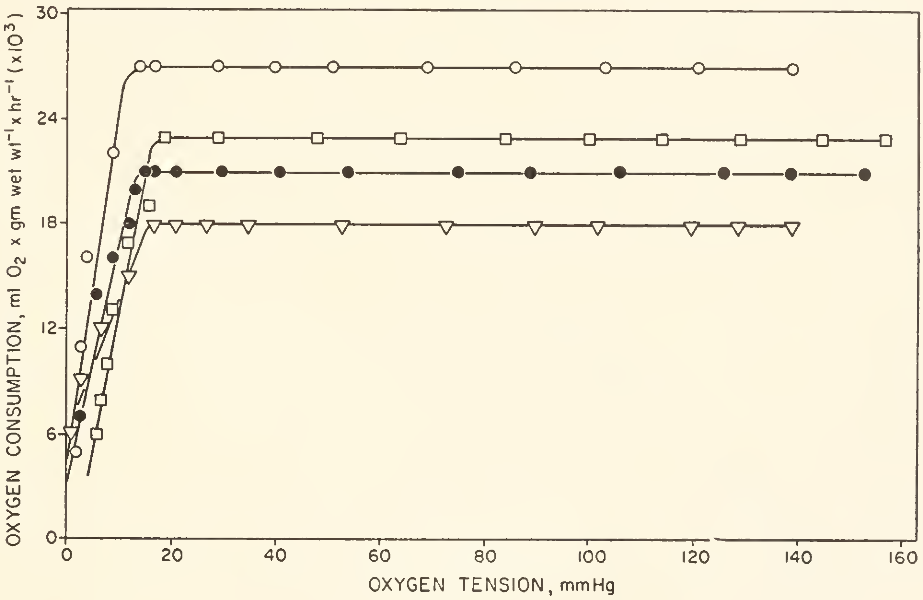

FIGURE 1. Oxygen consumption of *Callianassa californiensis* as a function of oxygen tension at 10° C (○ 7.3 g; □ 8.7 g; ● 5.7 g; ▽ 5.3 g).

RESULTS

Metabolic rate-oxygen tension measurements

The metabolic rates of four individual *Callianassa californiensis* in relation to oxygen tension are seen in Figure 1. Regulation occurs over the range of oxygen tensions from 160 to 20 mm Hg. Datum points are sufficiently different for each individual in this independent range of regulation to warrant separate curves. A distinct break in the metabolic rate curve occurs at 10–20 mm Hg. This range, the oxygen tension at which the metabolic rate ceases to be independent, is called the critical oxygen tension, or T_c (Prosser, 1955). Thus at a T_c of 10–20 mm Hg, corresponding to 0.4–0.8 ml O_2/l or 6.2–12.5% air-saturation, metabolism becomes directly dependent upon external oxygen concentration.

The metabolic rate *vs.* oxygen tension data of four *Upogebia pugettensis* are shown averaged in a curve drawn by inspection (Fig. 2). The metabolic rate is independent of external oxygen concentration as the tension is lowered from air-saturation to approximately 50 mm Hg. Compared to *Callianassa,* the T_c occurs at considerably higher oxygen tensions, 45–50 mm Hg, corresponding to 1.7–1.9 ml O_2/l, or *ca.* 30% air-saturation. No significance is attached to an apparent slight change in slope at approximately 15 mm Hg.

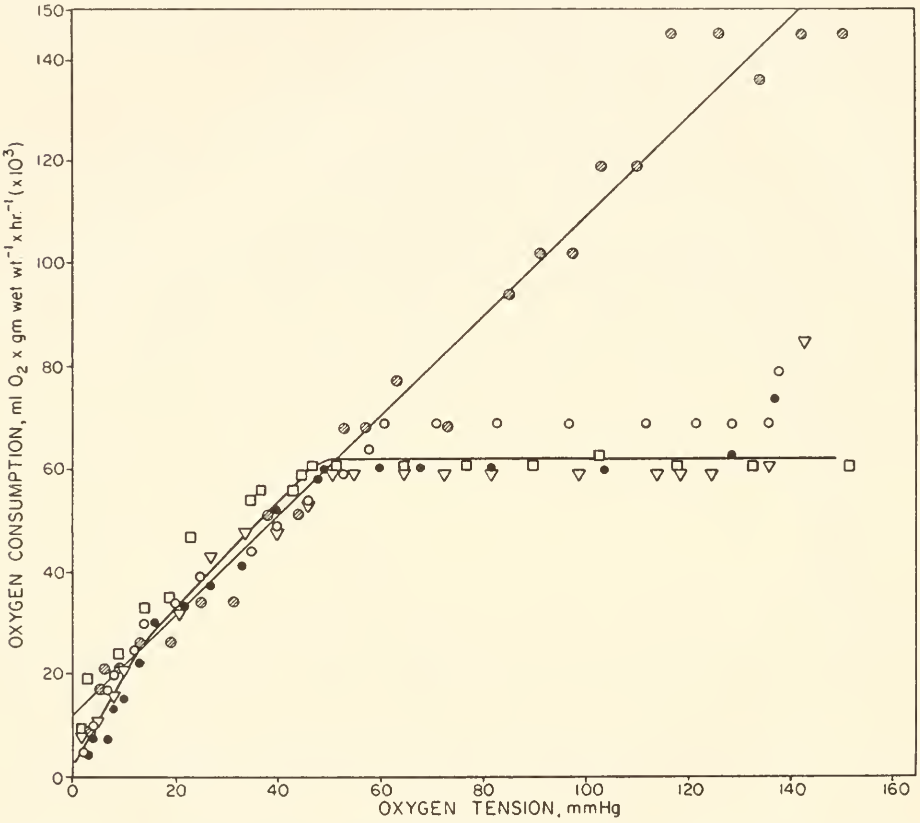

FIGURE 2. Oxygen consumption of non-molted *Upogebia pugettensis* (○ 6.5 g; □ 3.4 g; ● 8.2 g; ▽ 5.9 g) and of a postmolt *U. pugettensis* (shaded circles 3.7 g) as a function of oxygen tension at 10° C.

Metabolic pattern may be altered by experimental conditions. Hiestand (1931) reported that the crayfish *Orconectes* (=*Cambarus*) *virilis* (Hagen), normally a regulator with respect to metabolism, shows a conforming pattern if the jar-animal volume ratio is too small or if the experiment commences at less than air-saturation. In the present experiments consideration is given to this problem by using relatively large jar-to-animal volume ratios.

The metabolic rates of both species were determined at air-saturation. The average metabolic rate for *C. californiensis* ($n = 16$; mean wt 5.3 ±1.5 g) is

0.0291 ± 0.009 ml $O_2 \times$g wet wt$^{-1} \times$ hr^{-1}. *U. pugettensis* ($n = 8$; mean wt 5.7 ± 1.3 g), on the other hand, has a mean metabolic rate of 0.0599 ± 0.014 ml $O_2 \times$ g wet wt$^{-1} \times$ hr^{-1}, or twice that of *Callianassa.* The difference between the means is significant at the 1% level ($t = |6.67| \geqslant t_{.01} = 2.82$). Effects of activity on oxygen consumption were not measured in the present investigation. We believe that the rates reported above should be considerd as "routine" metabolic rates (as defined by Fry, 1957).

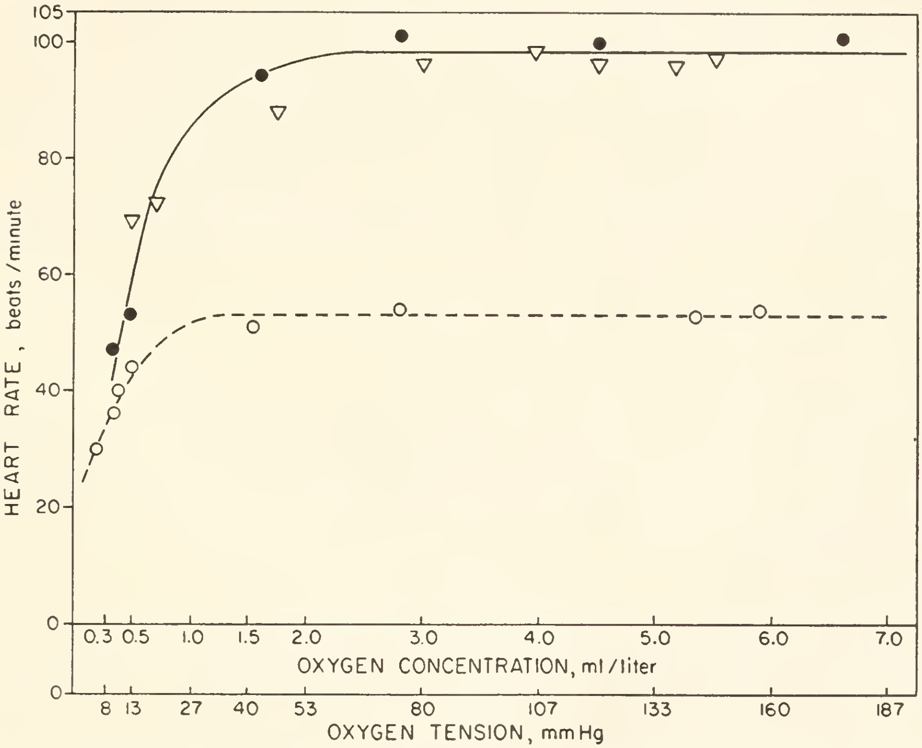

FIGURE 3. Heart rate of *Callianassa californiensis* in beats per minute at various oxygen tensions at 10° C; inactive shrimp ○, ●; active ▽; solid line represents two shrimp.

The metabolic rate curve, determined by liner regression, of a recently molted *U. pugettensis* is also seen in Figure 2. It is apparent that metabolic rate is directly dependent upon external oxygen concentration and that a regulatory phase is absent. This postmolt *Upogebia* has a notably greater respiratory rate at higher oxygen tensions than the non-molted *Upogebia;* however at tensions less than 45–50 mm Hg, metabolic rates are comparable.

A criticism of the sealed jar method used in the present study involves the possible effect of the accumulation of waste products as the animal depletes the available oxygen. However, the fact that shrimp were shown to survive in a sealed jar from three to six days (Table I) leads us to believe that accumulation of waste products in 10–20 hours probably has little effect on the animals.

TABLE I

Survival time in hours for Callianassa californiensis *and* Upogebia pugettensis *individually subjected to anoxic conditions. Averages are presented with the standard deviation*

Animal number	*Callianassa californiensis*			*Upogebia pugettensis*			
	Sex	Wet weight grams	Survival time hours	Animal number	Sex	Wet weight grams	Survival time hours
1	♀	2.8	156	1	♂	1.9	95
2	♂	4.0	111	2	♀	3.7	92
3	♂	4.1	128	3	♂	3.8	72
4	♂	4.3	156	4	♀	5.9	64
5	♂	4.8	108	Average of non-molted animals: 81 ± 15 hours			
6	♂	5.0	187	5	♀	5.5	12
7	♂	5.3	129	6	♂	5.6	42
8	♂	5.6	126	7	♂	7.4	43
9	♂	6.2	132	Average of postmolt animals: 32 ± 18 hours			
10	♂	6.4	179				
11	♂	7.2	110				
Average (all non-molted: 138 ± 27 hours							

Heart rate measurements

The effect of declining oxygen tension upon the heart rate of three *C. californiensis* is illustrated in Figure 3. A single curve for two of the animals has been drawn, while the data for the third animal are shown separately. Activity was operationally defined by movement of legs and pleopods and inactivity by movement of only the gill bailer.

TABLE II

Survival time in hours for non-molted Callianassa californiensis *subjected to anoxic conditions in groups of 12 shrimp per 3.8-liter jar*

Animal number	Group Study A			Group Study B			
	Sex	Wet weight grams	Survival time hours	Animal number	Sex	Wet weight grams	Survival time hours
1	♀	1.5	138	1	♂	2.0	179
2	♀	1.5	138	2	♂	2.1	78
3	♂	1.8	126	3	♀	2.1	129
4	♂	1.9	138	4	♀	2.2	141
5	♂	2.0	126	5	♀	2.3	179
6	♂	2.5	176	6	♀	2.6	129
7	♂	2.7	126	7	♀	3.0	129
8	♂	2.7	126	8	♂	3.2	78
9	♂	2.8	126	9	♂	3.2	78
10	♂	2.8	126	10	♀	3.3	179
11	♂	3.2	176	11	♂	3.4	129
12	♂	3.7	138	12	♂	3.6	78
Average: 138 ± 18 hours				Average: 126 ± 40 hours			

It is seen that heart rates vary considerably between 50 and 100 beats/min and that active and inactive rates overlap. However, it is clear that below *ca.* 1.5 ml O_2/l, the heart rate decreases rapidly with further decrease in tension. The decline in heart rates occurred near the metabolic T_c.

Survival time under anoxic conditions

The survival times for individually tested shrimp are seen in Table I. The average survival time for non-molted *C. californiensis* (138 ± 27hrs) is 1.7 times the average survival time for non-molted *U. pugettensis* (81 ± 15 hrs). However, the average survival time for postmolt *Upogebia* is only 32 ± 18 hrs. The survival times for *Callianassa* tested in groups of 12 (Table II) are essentially identical to those obtained for individually tested shrimp.

The results indicate that survival time under anoxia is independent of both sex and body weight. The possible effect of waste product accumulation and of pH change was not determined. Shrimp were not lethargic under anoxia but appeared to remain active until a few hours before death.

Burrow habitats

U. pugettensis builds relatively permanent burrows in a mud-clay substrate. The burrows were basically U-shaped with side branches, and extended downward to a depth of 2–3 feet. Each burrow had a minimum of two openings which were constricted near the surface and often surrounded by faecal pellets. These observations agree in general with those made by MacGinitie (1930).

On close examination the durable burrow walls, which were smooth and cylindrical in form, were coated with a reddish-brown layer 1–3 mm thick. Microscopic examination of this layer revealed that it is amorphous. In the laboratory the deposit occurred only in containers in which *Upogebia* had burrowed into the mud. Hence, it does not appear to be a function of the substrate alone. Pohl (1946) described a similar dark rust-brown lining (3–7 mm thick) in burrows of *Callianassa major*. Pearse (1945) briefly mentioned that burrows of *Upogebia affiinis* have firm linings like those of *C. major*. The exact nature of the lining and its formation is unknown. We conclude that *Upogebia* inhabits a relatively permanent burrow system with openings at the surface.

The concentration of dissolved oxygen in the water samples obtained from *U. pugettensis* burrows (Table III) at the time of low tide ranged from zero to 0.91 ml/l with an average of 0.58 ml/l. The average depth at which the samples were obtained was 60 cm. The average temperature within the burrows at 30 cm depth was 12.8 ± 1.7° C. Using each burrow water temperature and 33‰, the prevailing salinity of burrow waters throughout the summer, oxygen saturation values were estimated from nomograms (Richards and Corwin, 1956). According to these results burrow water samples were on the average 9.8% of full air-saturation, surface water 70.3% air-saturation, and aerated water in laboratory 97.5% air-saturation.

Burrows of *U. pugettensis* were located at the approximate zero to minus 1 ft tide levels and were thus exposed by the tide for less time than burrows of *C. californiensis* which occurred in higher intertidal areas (zero to +1 ft). Even so,

TABLE III

Amount of dissolved oxygen (ml/l) in water samples obtained from Upogebia pugettensis *burrows located between Coquille Point and Sally's Slough. Data were obtained on three different days at the time of low tide. Averages are given with the standard deviation*

Date	Burrow number	Depth cm	Temp. °C at 30 cm	ml O_2/l	% Air-saturation of burrow water
5/11/66	1a	51	12.0	0.77	12.9
	1b	66	12.0	0.77	12.9
	2	66	12.5	0.88	14.9
	3	66	12.5	0.41	6.9
	4	51	12.0	0.59	9.9
	5	66	12.5	0.88	14.9
5/24/66	6	61	13.0	0.70	12.1
	7	61	12.0	0.75	12.6
	8	61	11.0	0.91	13.5
	9	61	12.5	0.31	5.2
	10	61	11.0	0.62	9.2
	11	61	12.0	0.67	11.2
6/24/66	12	48	—	0.00	00.0
	13	56	16.0	0.39	7.1
	14	61	16.0	0.31	5.6
	15	61	16.2	0.39	7.1
Average		60 ± 6	12.8 ± 1.7	0.58 ± 0.26	9.8 ± 4.1

within one hour after exposure of the burrow openings by the ebbing tide, the oxygen concentration of water in each *Upogebia* burrow decreased by 46–100% (Table IV).

Salient features of *C. californiensis* burrows in the Yaquina Bay region are reported by L. C. Thompson and Pritchard (1969). It is concluded that burrows of *Callianassa* are not firmly constructed, lack a lining, and generally do not have patent openings to the surface during ebb tide.

TABLE IV

Amount of dissolved oxygen (ml/l) in water samples taken from randomly selected Upogebia pugettensis *burrows on May 26, 1966. Each burrow was sampled twice as the tide ebbed. Average sampling depth was 62 ± 3 cm and average temperature was 10.6 ± .4° C*

Burrow number	Burrow diameter cm	Time hours	ml O_2/l	Per cent change
1	1.9	0900	2.70	72
		1000	0.75	
2	1.3	0915	2.18	
		1015	0.00	100
3	1.9	0930	2.44	
		1030	0.99	55
4	2.5	0940	1.56	
		1040	0.83	46
5	1.9	0950	1.14	
		1050	0.36	68

Four interstitial water samples were taken at low tide from the collecting area of *Callianassa* (0.46, 0.58, 0.81 ml O_2/l) and one from the collecting area of *Upogebia* (0.15 ml O_2/l).

Discussion

Callianassa californiensis and *Upogebia pugettensis* at 10° C are metabolic regulators with T_c's far below air-saturation, have low metabolic rates, and are remarkably tolerant to anoxic conditions. Critical oxygen tensions have not been determined for other members of the Thalassinidea. For references on other decapod crustaceans with a T_c below air-saturation see Wolvekamp and Waterman (1960). Mean metabolic rates, within the range of respiratory independence, of *Callianassa* (0.029 ml $O_2 \times$ g wet wt$^{-1} \times$ hr^{-1}) and of *Upogebia* (0.059 ml $O_2 \times$ g wet wt$^{-1} \times$ hr^{-1}) are comparable to the metabolic rates of other mud-dwelling forms such as the polychaete *Arenicola,* the oligochaete *Enchytraeus* and the echiuroid *Urechis,* 0.031, 0.030, and 0.012 ml $O_2 \times$ g wet wt$^{-1} \times$ hr^{-1}, respectively (Prosser and Brown, 1961). Montuori (1913) reported respiration rates of 0.132 and 0.368 ml $O_2 \times$ g wet wt$^{-1} \times$ hr^{-1} at 25° C for *Callianassa subterranea* and *Gebia litoralis,* respectively. Such comparisons, however, have limited significance since experimental conditions in the various investigations differed greatly.

Mudflats have long been recognized as environments impoverished with respect to oxygen (Brafield, 1964; Pearse *et al.*, 1942; ZoBell and Feltham, 1942). Sand or mud substrates greatly impede rapid exchange of oxygen with the overlying waters. Such oxygen as is available is generally restricted to the upper surfaces and to portions of the substrate in contact with overlying oxygenated water via specialized tubes, burrows, *etc.* Tidal exposure may interrupt this exchange altogether. Below the top few centimeters of mud bacteria are largely responsible for anaerobic conditions, hydrogen sulfide, and a highly reducing environment.

In the Yaquina Bay estuary *C. californiensis,* unlike *U. pugettensis,* occupies the higher intertidal levels of the mudflats and is without a permanent burrow system. Burrows of *Callianassa* in the high intertidal area were often uncovered during a low high tide whereas burrows of *Upogebia* were not then exposed. As the tide ebbed, the upper parts of the impermanent sandy burrows tended to collapse and any connection with the overlying oxygen-rich waters was broken. Attempts to trace the burrows and to obtain burrow water samples during ebb tide were unsuccessful. The hypoxic nature of the habitat of *Callianassa* is shown by the interstitial water samples obtained. *Callianassa* does not depend on a water current in its burrow for food but instead sifts the substrate for detritus, burrowing continuously in order to feed (MacGinitie, 1934). Hence, in the absence of a permanent burrow system *Callianassa* is constantly exposed to hypoxic interstitial waters.

The metabolic responses of *C. californiensis* are adaptive to its survival in this hypoxic environment. *Callianassa* is capable of regulating its metabolic rate down to an extremely low T_c. Heart rate appears comparably regulated. Significant bradycardia did not occur until the oxygen tension had dropped below 27 mm Hg. Perhaps the maintenance of a constant heart rate and presumably constant cardiac output enables the shrimp to regulate its metabolic rate as the external medium becomes increasingly hypoxic. Both thalassinids, particularly *Callianassa,* are remarkably resistant to anoxia under laboratory conditions.

A thorough investigation of the mechanisms involved in tolerance of anoxia was beyond the scope of this study. However, several experiments to determine if a compensatory increase in metabolic rate occurred after subjection of shrimps to anoxia were performed. In both *C. californiensis* ($n = 2$) and *U. pugettensis* ($n = 4$) the oxygen consumption following exposure to either 12 or 36 hours of anoxia increased above the pre-anoxic rate. These preliminary experiments suggest that anaerobic metabolism is used by these shrimp during anoxic stress. However, the nature of the anaerobic pathway or pathways, the magnitude of the oxygen debt after longer periods of anoxia, and the metabolic products produced remain to be elucidated. Although von Brand (1946) pointed out that crustaceans, especially decapods, show little tolerance for anoxic conditions, there is more recent evidence for anaerobic metabolism in decapods. Teal and Carey (1967) report an increase in lactate and subsequent decrease in glycogen content in *Uca* under anoxic conditions.

In a recent report Farley and Case (1968) demonstrate "ventilation" behavior by *C. affinis* and *C. californiensis* in response to altered oxygen tension and hypothesize the existence of an oxygen receptor, the direct evidence for which is presently lacking. Comparisons between the species are difficult because of the complex behavioral responses and the lack of comparable experimental conditions for the two species. Thus, under one set of conditions *C. affinis* responded to lowered oxygen and to readmission of oxygenated SW by increasing the stroke frequency of the pleopods and under other conditions *C. californiensis* after experiencing a limited hypoxia migrated toward oxygen-rich SW and increased the stroke frequency of the pleopods.

In the Yaquina Bay estuary water samples from the burrows of *Upogebia* at low tide are markedly hypoxic, becoming more so as the period of tidal exposure increased (Tables III, IV). The data also indicate that during tidal ebb *U. pugettensis* experiences oxygen concentrations well below its T_c; occasionally the oxygen concentration drops to zero. The same may be presumed true for interstitial waters of mud, although the data are more limited. In the present study the mean concentration of oxygen in burrow waters of *Upogebia* is 0.58 ml O_2/l. Only one reliable interstitial water sample from the *Upogebia* collecting area could be obtained with the sampling device described (0.15 ml O_2/l).

Upogebia shows physiological and ecological adaptations to these hypoxic conditions. Its metabolic rate is among the lowest reported for Crustacea (Wolvekamp and Waterman, 1960). Non-molted shrimp regulate metabolism above 45–50 mm Hg and can tolerate more than two days without oxygen, which at the zero tide level far exceeds the maximum tidal exposure of Pacific coast mudflats (MacGinitie, 1935). The permanent burrow system and its apertures represent open channels for exchange with the oxygen-rich overlying waters. More important, *Upogebia* actively irrigates its burrows, and as a suspension feeder, is dependent upon such irrigation (Jørgensen, 1966; MacGinitie, 1930). As a consequence *Upogebia* probably experiences in its burrows higher oxygen concentrations than those of neighboring interstitial waters, as is true for other tube-dwellers in hypoxic habitats. For instance, the burrow waters of the polychaete *Arenicola* have an oxygen concentration (0.50 ml O_2/l), twice that of nearby interstitial waters (Jones, 1955). The tube material of a sedentary polychaete, *Mesochaetopterus taylori*, apparently acts as a protective diffusion barrier to oxygen, hence the tube waters contain significantly more oxygen than do the surrounding anoxic interstitial waters (Petersen and Johansen, 1967).

Information about the effect of molting on the respiration of crustaceans is limited but it is generally agreed that respiration increases at the time of ecdysis (Passano, 1960). The present data suggest that postmolt *Upogebia* are relatively more oxygen-dependent and less resistant to anoxia than are non-molted animals. Nothing is known about the behavior of *Upogebia* during molting under natural environmental conditions.

Metabolic requirements, indicators of which are T_c, metabolic rate, and tolerance to anoxia, generally reflect oxygen availability in the environment. Animals inhabiting environments high in oxygen usually have greater metabolic requirements than those inhabiting environments low in oxygen. Isopods, ephemerid nymphs and trichopterid larvae from ponds have lower metabolic rates, survive longer in low oxygen and have lower T_c's than the same or related species from swift streams (Fox and Simmonds, 1933; Fox, Wingfield and Simmonds, 1937). Walshe (1948), emphasizing that differences in metabolic requirements reflect oxygen availability in the habitat, reported that stream chironomid larvae (metabolic conformers) have higher metabolic rates and are less resistant to hypoxia than those from ditches (metabolic regulators). Bovbjerg (1952) found that *Cambarus fodiens,* a mud-burrowing crayfish of ponds, survived anoxic conditions four times longer than *C. propinquus,* an inhabitant of swift streams.

In conclusion, emphasis is placed on the adaptive significance of the respiratory responses reported for mud-dwelling thalassinids in the present study. *C. californiensis* and *U. pugettensis* live in mudflats, an environment relatively low in oxygen. Both show the following physiological mechanisms: (1) low metabolic rates; (2) metabolic regulation with a T_c below 50 mm Hg; and (3) survival in anoxia for at least three days. These mechanisms correlate well with a hypoxic habitat and are therefore considered adaptive. Closer analysis reveals quantitative differences in their respiratory responses. *Upogebia* has a greater metabolic rate, higher T_c, and is less able to tolerate anoxia than *Callianassa.* Despite the paradoxical situation of living in a substrate poorer in oxygen, *Upogebia* probably has in fact more oxygen available in its specific niche within the mudflat habitat than does *Callianassa;* hence, metabolic requirements of *U. pugettensis* are greater and regulatory features are less pronounced than in *C. californiensis.* The present study supports the generality that metabolic requirements and the availability of oxygen in the environment are closely correlated.

Sincere appreciation is expressed to the staff of the Oregon State University Marine Science Laboratory. We would also like to thank R. I. Smith, J. W. Born and L. C. Thompson for their critical reading of the manuscript. R. K. Thompson wishes to thank Ivan Pratt who made her stay at the Marine Laboratory possible by providing a resident fellowship.

SUMMARY

1. The respiratory responses of two mud-shrimps, *Callianassa californiensis* and *Upogebia pugettensis* (Thalassinidea), from Yaquina Bay, Newport, Oregon, were measured.

2. Both species are metabolic regulators, showing oxygen-independent respiration above the critical oxygen tension for *C. californiensis* of 10–20 mm Hg (6.–12.5% air-saturation) and for *U. pugettensis* of 45–50 mm Hg (28–31% air-saturation).

3. Within the independent range of respiration, *C. californiensis* has a mean metabolic rate of 0.029 ml $O_2 \times g$ wet $wt^{-1} \times hr^{-1}$, which is significantly lower than that of *U. pugettensis* (0.059 ml $O_2 \times g$ wet $wt^{-1} \times hr^{-1}$).

4. Heart rates of *C. californiensis* subjected to diminishing oxygen tensions show a regulatory pattern similar to the metabolic rate, with bradycardia occurring at *ca.* 27 mm Hg.

5. Both species are tolerant to anoxia. *C. californiensis* survives approximately 5.7 days and *U. pugettensis* 3.3 days under such conditions.

6. Preliminary data suggest that postmolt *U. pugettensis* do not regulate and therefore are oxygen-dependent throughout the range tested.

7. The mean concentration of oxygen in water obtained from exposed *U. pugettensis* burrows is 0.58 ml O_2/l, well above that of interstitial water.

8. *C. californiensis,* in contrast to *U. pugettensis*, does not construct firm burrows and is probably directly exposed to hypoxic interstitial waters.

9. Both species have respiratory adaptations for survival in a hypoxic environment. Quantitative differences in the metabolic requirements of the two species reflect the availability of oxygen in their respective niches within the mudflat biotope.

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LEG EXTENSION IN *LIMULUS*

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The ancestors of arthropods were probably worm-like animals with hydrostatic skeletons. With the evolution of a rigid exoskeleton the hydraulic system lost importance as a means of support and leg extension and has apparently disappeared in most adult arthropod forms. Nevertheless hydraulic systems seem to persist in a few groups; such animals typically show relatively large membranous areas where the integument is not stiff, and lack extensor muscles in one or more of their leg joints. Spiders, various myriapods, scorpions, and *Limulus* show both of these characteristics. These characteristics alone, however, are not sufficient evidence for the presence of a hydraulic system. Only in spiders is the existence of such a system well substantiated (Parry and Brown, 1959a, 1959b). In centipedes, millipedes, and scorpions the evidence for the presence of hydraulic leg extension is equivocal (Manton, 1958a). We have no evidence other than the above characteristics that a hydraulic system exists in *Limulus.* The problem is especially interesting in *Limulus* since the horseshoe crab is considered important in discussions of arthropod evolution.

The femoro-patellar joints of all the walking legs of *Limulus* (*Limulus polyphemus*) are hinge joints lacking extensor muscles (see Fig. 1). Nevertheless, in the power strokes of normal walking the femoro-patellar joints of *Limulus* perform up to 120 degrees of extension from the flexed state. This is the greatest angular displacement occurring at any joint in the legs. The mechanism of extension of these joints is not known. These extensions are important in performing walking motions of the legs, which propel the animal over intertidal mud flats and through surface sediments of the ocean floor.

In this paper I present data concerning femoro-patellar extension in *Limulus.* I propose and provide evidence for a mechanical scheme of extension of these extensorless hinge joints. I conclude that the mechanism is neither hydraulic nor elastic.

EXPERIMENTAL ANIMALS

Live specimens of *Limulus* were obtained commercially from Panacea, Florida, and collected at Beaufort, North Carolina. The animals used for pressure measurements ranged from 20.0 to 23.5 cm in length from the front of the carapace to the base of the telson. Larger or smaller animals were used for other work. Some animals arrived in poor condition, showed below normal activity and died a few days later. These animals also showed below normal blood pressures that were as low as zero in some cases; data from such animals are not included here. In one *Limulus* the blood pressure was very low but the animal appeared otherwise normal; these data are therefore included. All animals included became quite active when picked up and they lived in the laboratory for three or more weeks even after various operations. The animals were kept in tanks containing aerated artificial sea water ("Instant Ocean," Aquarium Systems Inc., Wickliffe, Ohio) at 13° C.

NORMAL HINGE JOINT ANGLES

The maximum *in vivo* change in angle from the flexed position of the femoro-patellar joint ranged from 35 to 71 degrees provided the limb was not pushing against any object. This range of extension was determined from measurements (using a protractor) on each of several legs of six animals in both inverted and upright positions. When the limb had a purchase distally, as on the ground in walking, extension of up to 120 degrees from the flexed position was observed. Some experiments were done with the limb tips off the ground and some with the limb tips on the ground. In all cases the angles obtained at the femoro-patellar joint were compared only to the angles of this joint in the intact animal in the same position.

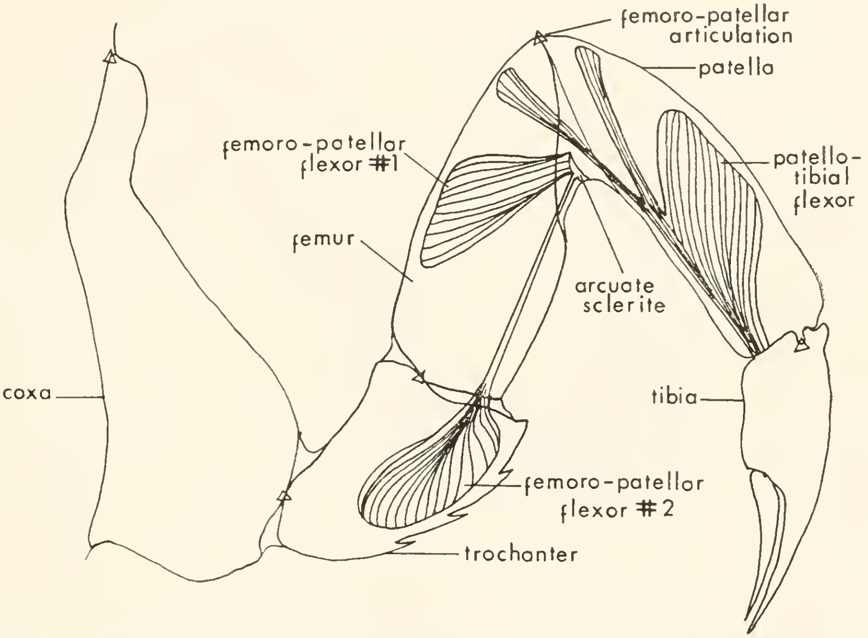
femoro-patellar articulation
patella
femoro-patellar flexor #1
patello-tibial flexor
femur
arcuate sclerite
coxa
tibia
femoro-patellar flexor #2
trochanter

FIGURE 1. Muscles at the femoro-patellar joint of left leg V of *Limulus*. Strong flexors are attached to the arcuate sclerite in the ventral membrane of the joint. The two slender muscles attached near the joint are part of the patello-tibial system. Contraction elicited by electrical stimulation of each muscle near the femoro-patellar joint failed to extend this joint. Morphology of all the structures shown here is very similar in all the legs. Points of articulation are marked with △.

DENERVATION

Methods

If extension of the femoro-patellar joint were done by muscles, then denervation of all the leg muscles should eliminate the extension. The main leg nerve (the only nerve entering the leg) was cut at the base of the coxa in intact animals with as little damage to the rest of the tissues as possible. In other animals the nerves were cut at other sites to localize the elements that performed the extension, as follows. Starting distally the nerve was cut at every joint in the leg. After each cut the minimum and maximum extensions of the femoro-patellar joint were determined. The maximum and minimum angles were previously measured in the intact animal. In every case the same operation was performed on the contralateral leg without cutting the nerve; this operation acted as a control for factors such as injury or loss of blood that might impair extension or activity in general.

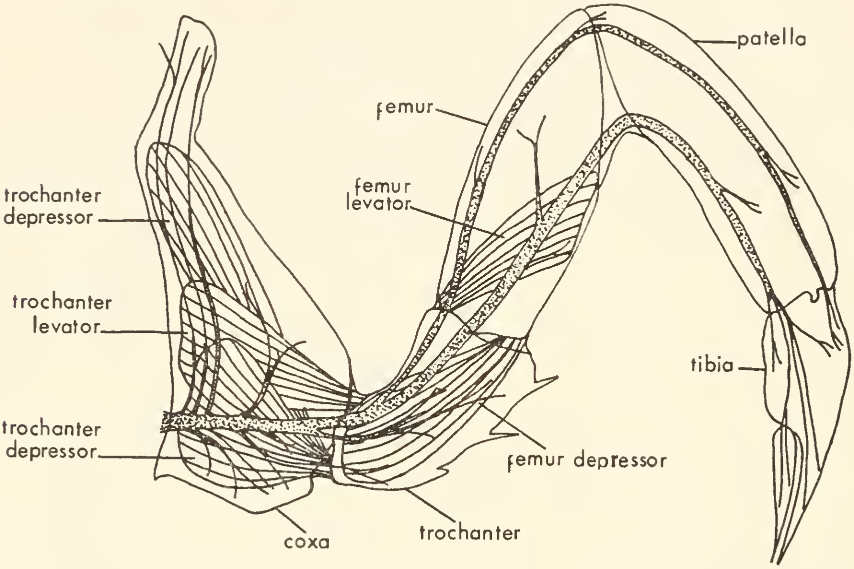

FIGURE 2. Nerves in left leg V of *Limulus,* anterior view. All structures shown here have very similar analogs in all walking legs. The nerves are shown in heavy stippling (detail added from Patten and Redenbaugh, 1899). The muscles shown are only those found in the coxa and trochanter (femoro-patellar flexor #2, which inserts in the trochanter, has been excluded: see Fig. 1). For musculature of the femur and patella, see Figure 1.

In the segments where the small leg nerve is also present (the main leg nerve splits into two branches in the trochanter; see Fig. 2), both nerves were cut to eliminate all innervation of the muscles in that segment.

Results

When all leg musculature was denervated by cutting the nerve at the base of the coxa no measurable extension of the femoro-patellar joint occurred. This indicated that some muscles were involved in the extension of this hinge joint. Since the above denervations were the only ones that completely eliminated extension of the femoro-patellar joint, muscles in the coxa must be involved in the extension of the hinge joint. When the nerve was cut at the coxa-trochanter joint (see Fig. 2), zero to 96% of the maximum *in vivo* extension was observed, indicating that the muscles in the trochanter may also have a role in the extension. Denervation at sites other than the prosoma-coxa and coxa-trochanter joints in no way affected the extension of the femoro-patellar joint. A flexor of the femoro-patellar joint also attaches in the trochanter; however, cutting this muscle or the nerves that innervate it did not decrease the extension of the femoro-patellar joint.

MUSCLE STIMULATION

Methods

To identify possible muscular effectors of extension various muscles and muscle groups were stimulated electrically in excised legs, simultaneously measuring changes in femoro-patellar joint angle. The excised legs were placed in a position similar to the normal flexed position in the live *Limulus*. "Windows" were cut in the exoskeleton of each segment of the leg, leaving the muscles (except for the fasciae attached directly to that portion of the exoskeleton), joints and other structures intact. The muscles were then stimulated through the windows. Stimulation was done with silver wire electrodes and a square wave or induction stimulator at 20 volts. This experiment was done with the limb tip free and with the limb tip fixed (allowing movement at the proximal end in the case of the limb tip fixed).

Results

Normal extension of the femoro-patellar joint, both for the limb tip free and the limb tip held fixed, was produced only by contraction of the depressors of the trochanter or the depressors of the femur or both (see Fig. 2). The contraction of no other muscle produced extension of that hinge joint under these conditions.

OBSERVATIONS CONCERNING ELASTICITY

Methods

Excised legs were examined for a possible elastic source of hinge joint extension as follows. Excised legs were flexed manually to various angles and released after each forced flexure. Any changes in femoro-patellar joint angles following the flexures were recorded. This experiment was done on freshly excised legs with the muscles intact and was repeated after cutting the flexors of the femoro-patellar joint. The same tests were done for various orientations of the leg with respect to gravity.

The dorsal membrane of the hinge joint was pulled at either end to determine any changes in length, as measured by a millimeter ruler, resulting from tensile stress.

Results

No recoil, that is, no change in hinge joint angle, occurred after forced flexure of the joint in any case within the range of angles observed *in vivo*. There was no change in length measurable by the method used in the dorsal membrane of the hinge joint. No other structures were found which seemed in any way capable of elastically extending the femoro-patellar joint.

PRESSURE MEASUREMENTS

Methods

Measurements of pressure were made with a pressure transducer connected to a catheter fitted with an 18-gauge hypodermic needle at the other end. The transducer was a Sanborn model 267B physiological pressure transducer; it was coupled to a Beckman Offner type RS Dynograph amplifier-recorder. The transducer and catheter were filled with saline for fluid-system pressure measurements. The readings were taken with the needle inserted into the haemocoel through an arthrodial membrane or a hole drilled in the exoskeleton. Possible interference by clotting was avoided by using a hypodermic needle sleeve which was cleared with a metal core before inserting the needle. After each measurement the transducer was checked with a known pressure and compared to the initial calibration; if the results of the two calibrations were not identical, the data from that measurement were discarded.

Measurements on live specimens of *Limulus* were made at various sites in the legs, including under the membrane of the femoro-patellar and tibio-tarsal joints and under the exoskeleton of the femur and of the patella. Blood pressure was also measured dorsally through the carapace in the region of the heart, and ventrally through the membranes of the prosoma (cephalothorax).

Results

The average prosomal pressure at rest for eight specimens of *Limulus* was 14.1 mm Hg ± 11.6 (mean ± standard deviation). The average prosomal pressure during activity for four specimens of *Limulus* was 23.8 mm Hg ± 16.5. The comparison between these overall means does not show a statistically significant difference ($t = 1.23$, $0.20 > p > 0.10$). However, the difference between resting and active mean pressures is statistically significant in every case if the comparison is made for each animal ($t = 2.93$, $0.01 > p > 0.005$ in one case, and the means were separated by more than two standard deviations in all other cases).

The activities included in the active measurements were struggling by the legs, flapping of the gills, and flexion and extension of the prosoma-opisthosomal joint.

Most measurements were made ventrally through the membranes of the prosoma; four measurements were made dorsally in the region of the heart. The latter values were all within the range of the pressures measured ventrally in the same animal.

The *in vivo* pressure in the legs did not change significantly with extension of the femoro-patellar joint. For seven specimens of *Limulus* the mean pressure in the legs while the femoro-patellar joint was fully flexed was 5.1 mm Hg ± 1.8; while the same joint was extended the mean pressure was 6.2 mm Hg ± 2.4. When these means are compared the difference is not statistically significant ($t = 0.97$, $0.15 > p > 0.10$). Comparisons of pressures in flexed and extended legs for individual animals show statistically significant differences in only three out of eight cases. Pressures recorded while the femoro-patellar joint was in intermediate positions were between the flexed and extended values.

Determination of Pressure Required for Extension

Methods

The pressure required to extend hydraulically the femoro-patellar joint of a detached leg was determined by the following procedure. Legs were excised from nine live specimens of *Limulus* at the base of the trochanter; the leg haemocoel was then closed off with a rubber stopper (see Fig. 3) and the internal pressure was then increased by injecting saline with a syringe into the closed system. Simultaneously, the internal pressure in the leg was monitored and the change in femoro-patellar joint angle was measured with a protractor. Measurements of the pressure through the membrane of the hinge joint and through the exoskeleton gave the same results as measurements through the stopper. Subsequent measurements were therefore made through the stopper, which was more convenient.

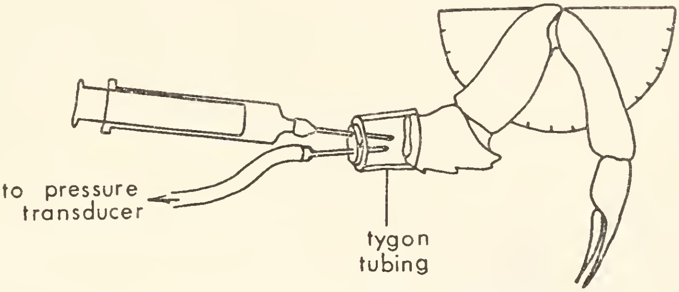

Figure 3. Determination of pressure required for extension.

Results

The pressures measured *in vivo* in the legs are much smaller than the pressures required to extend the hinge joint hydraulically. Figure 4 shows the pressure required for extension of the femoro-patellar joint determined for nine animals. The pressure needed to produce the observed extension of about 50 degrees in this position was about 150 mm Hg according to this graph. Also on Figure 4 are the mean *in vivo* leg blood pressures. These means are the normal blood pressures described above, measured *in vivo* in the leg while the femoro-patellar joint is fully flexed and while it is fully extended. The mean leg blood pressures are only enough to account for about a three-degree change in joint angle. In no case did the *in vivo* leg pressures reach a value that could account for a ten-degree extension as determined by the graph, whereas normal extensions by the animal ranged from 35 to 71 degrees under these conditions.

Cutting all the flexors reduced the pressure needed for full extension of the hinge joint by only 8%. Thus any overestimation of the pressure needed for extension due to overcoming the friction or the force of contraction of the flexors amounted to at most 8%. In fact, the error from this source was probably much smaller since in the living animals the force of extension must overcome some resistance in the flexors even when these muscles are relaxed.

DISCUSSION

Indirect muscular mechanism

The results of denervation and muscle stimulation and the observation of walking movements in live specimens of *Limulus* support the hypothesis that extension of the femoro-patellar joint is performed by a simple mechanical system, the effectors of which are the depressors of the trochanter, the depressors of the femur, and the coxal remotors (term of Manton, 1964). With this system, all observed extension movements of the extensorless femoro-patellar joints of *Limulus* can be explained. The various cases of extension observed in the intact animal are described below; following each is an explanation of the movement in terms of the proposed mechanical scheme.

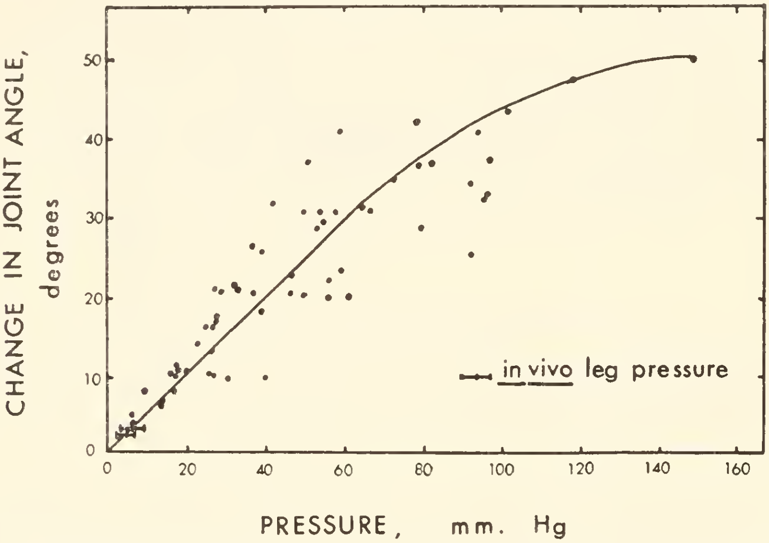

FIGURE 4. Extension of the femoro-patellar joint with artificial increase in internal hydrostatic pressure. The *in vivo* leg pressures are also shown (mean and one standard deviation on either side of the mean) to show contrast between the pressures required to extend the leg and the pressure actually found in the leg.

The first four pairs of walking legs (prosomal appendages II through V) may perform up to 70 degrees of extension of the femoro-patellar joint when off the ground. When the animal is upside-down in air, the femoro-patellar joint usually extends about 50 degrees as the limbs are pushed upwards, until a point is reached when the patella and more distal segments fall inwards to the normal flexed position. These movements can be effected by the action of the depressors of the trochanter and of the femur, which push the leg upwards in this position, and by the weight of the patella and more distal segments, which causes the femoro-patellar joint to extend (see Fig. 5a). Once the patella and more distal segments reach the point where their weight tends to pull them inwards (Fig. 5b), they fall passively over into the flexed position. These extension movements and those described below require previous relaxation of the flexors of the femoro-patellar joint.

In the normal upright position, the first four pairs of walking legs also may extend when the legs are off the ground. In the recovery strokes of walking, the femoro-patellar joint extends about 30 degrees, at which point the patella and more distal segments are in a vertical position. No further extension occurs in the forward or recovery strokes of walking. These movements may be performed by the depressors of the trochanter and femur, again, bringing the entire leg downwards (see Fig. 6a): after relaxation of the flexors of the femoro-patellar joint, the depression of the basal segments of the limb and the weight of the patella and more distal segments would extend the femoro-patellar joint (see Fig. 6b).

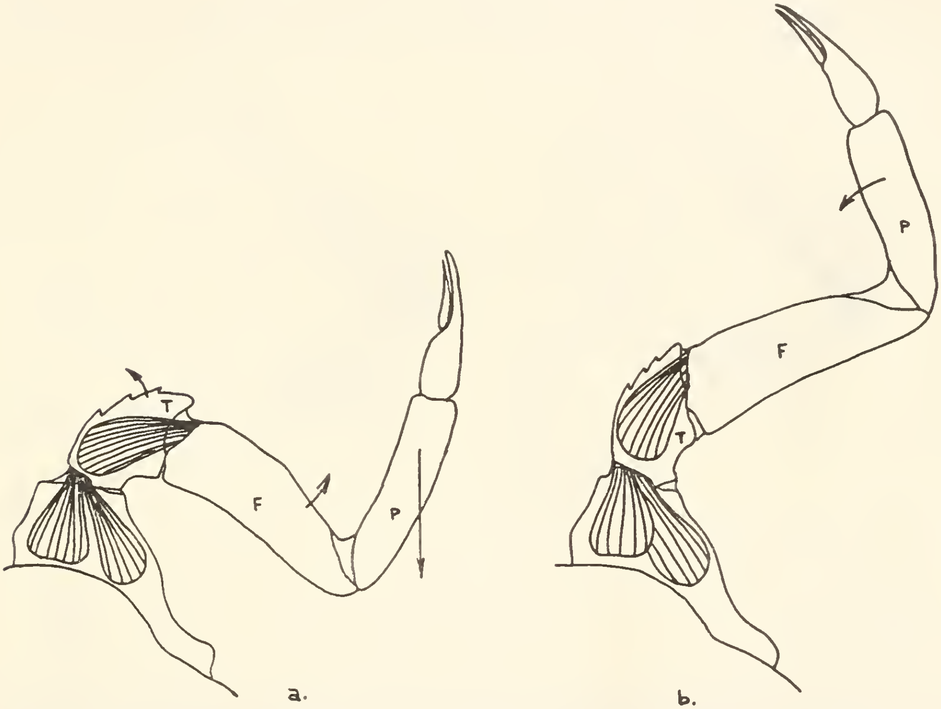

FIGURE 5. Extension of right leg V (anterior view) when the animal is upside-down. In this diagram the only muscles shown are those thought to extend the femoro-patellar joint. a: leg beginning extension. b: leg extended, patella and more distal segments about to fall into the flexed position. T = trochanter, F = femur, P = patella.

When the limbs are on the ground, the same movements are observed and the same explanations may be applied, with a few additions. When legs II through V have a purchase distally they can perform additional extension of the femoro-patellar joint (up to 120 degrees). As the depressors push the leg down, the resistance of the ground at the limb tip causes forward or upward movement of the body and is accompanied by extension of the femoro-patellar joint. Also, the depressors of the tibia must be contracting to keep the patello-tibial joint rigid as the leg bears down on the ground and the hinge joint extends. The same "postural" contractions must occur at most hinge joints whose muscles are not direct effectors of the movements being performed in walking.

The last pair of legs (prosomal appendages VI) has so far been excluded from the above descriptions because of its special movements. Even when these legs are off the ground, they may perform up to 120 degrees of extension of the femoro-patellar joints, provided the legs are positioned almost horizontally (see Fig. 7). The legs are kicked backwards, resulting in a straight-line configuration of all the segments in the legs. This results in a nearly 180-degree angle at the extensorless femoro-patellar joint, or a total extension of 120 degrees from the flexed position for most sixth legs. The same movement occurs when the animal is upside-down. The backwards rotation of the coxa is doubtless due to the coxal remotor muscles. Since the leg is now in a horizontal position, the axes of the joints are in a vertical position; therefore the weight of the segments beyond the femoro-patellar joint does not tend to flex that joint. The other pairs of legs also have coxal remotor muscles; however, the motions just described are not performed to any significant extent because rotation of the coxa is limited by the closely following coxae of more posterior legs. Note the position of the coxa of leg V in Figure 7b(ii).

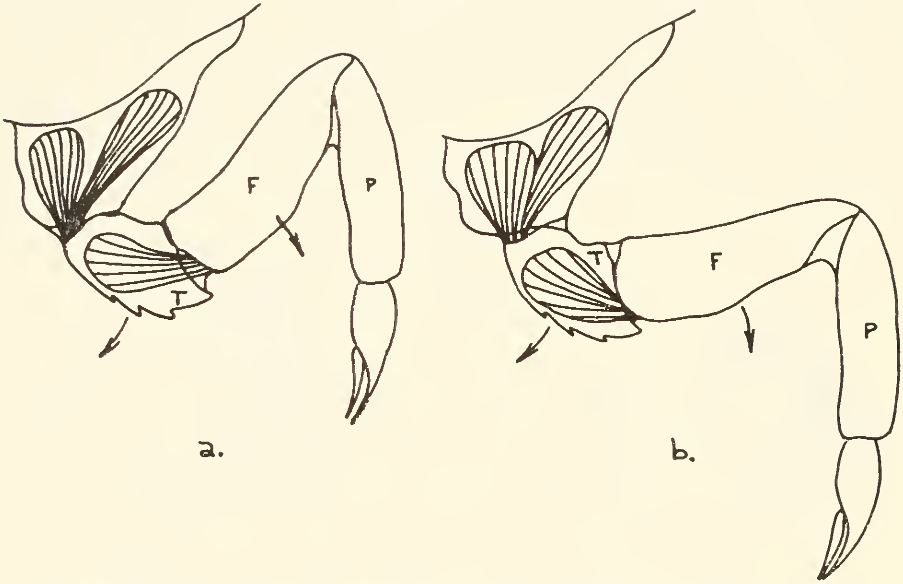

FIGURE 6. Extension of left leg V (anterior view) when the animal is in the normal upright position. The only muscles shown are those thought to effect the extension of the hinge joint. a: leg elevated. b: leg depressed and extended. T = trochanter, F = femur, P = patella.

The last pair of legs are the legs which seem to do most of the work in propelling the animal through mud and sand by rapid straightenings and application of the large area of their distal flattened spines to the substrate. The locomotion of *Limulus* through mud or on dry ground can be observed to proceed in two phases. There is a period of motion, then a pause, and the cycle repeats itself. This pattern is due in the movement phase to the power stroke of the rear legs and in the pausing phase to the recovery stroke of the rear legs. The other legs also perform stroking movements in walking, but these strokes do not always coincide with phases of movement of the animal. Since the tips of these legs are chelae instead of flattened plates, they can get little purchase in mud or sand unless there are configurations in the substratum which can be grasped by a chela. Thus most of the forward locomotion in *Limulus* is effected with the last pair of legs.

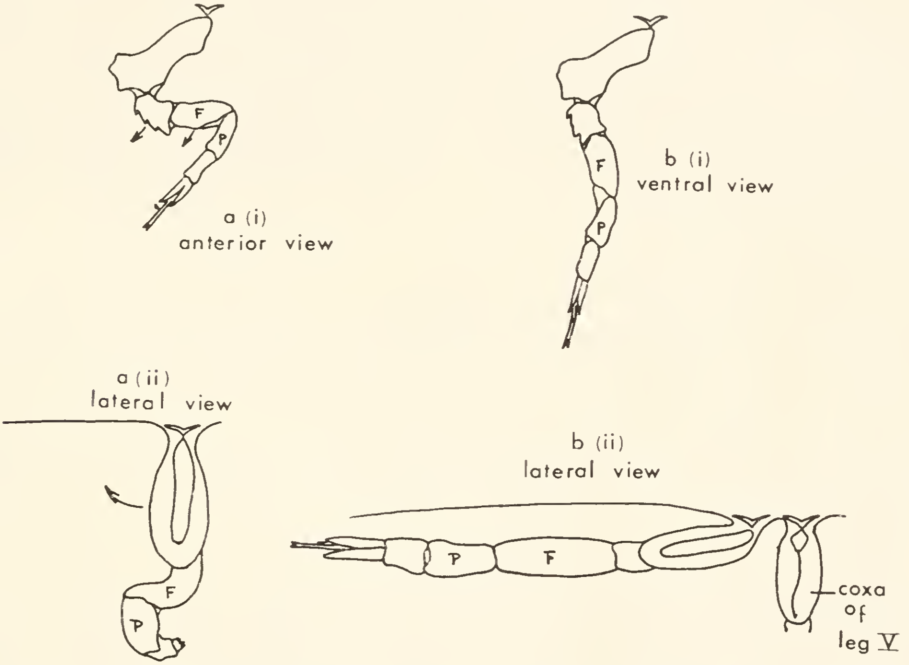

FIGURE 7. Extension of left leg VI. a: leg in normal flexed position. b: leg fully extended. The anterior view shown in a(i) is before rotation of the coxa; once rotated, as in b(i), the same surface becomes ventral. F = femur, P = patella.

The extension movements of the last pair of legs, when their tips are on the ground, are basically those observed in the same legs when their tips are off the ground; that is, the coxa rotates posteriorly, the legs are kicked back, and the hinge joint extends 120 degrees as the body moves forward. Thus the same mechanism may be applied to explain these extension movements.

The schemes that Manton (1958a, 1958b) outlined for myriapods, scorpions, and spiders are similar to that presented here for *Limulus*. However, in myriapods and scorpions rotation of the hinge joint surface occurs during extension of those joints in all the legs. These animals show varied and intricate morphological specializations for leg rotation (Manton, 1958a, 1958b). In *Limulus,* only the last pair of legs performs considerable rotation; and the weight of the leg is an important effector of extension of the hinge joints of all the legs.

Elasticity

The evidence against an elastic mechanism of femoro-patellar extension comes from several sources. In an excised leg no recoil occurs after a forced flexion as reported in the results, whether or not the muscles are cut. Thus neither the muscles nor the dorsal hinge joint membrane seem to provide energy for extension. Also, the dorsal hinge joint membrane does not stretch visibly when pulled; thus there is no evidence that it is a storage site for elastic force causing extension.

Snodgrass (1952) proposed that in the case of the prosoma-coxal joints of *Limulus,* elasticity, either of the muscles or the membranes, may have a role in extension, but there is no evidence available to explain or support its existence. Manton (1964) proposed that there are extensors in those joints of *Limulus.* The muscles she describes as extensors are the two attaching on either side of and nearest to the pleurocoxal articulation. In any case, these hinge joints perform little extension even during feeding movements, which involve motions of the gnathobases of the coxae.

Hydraulic mechanism

Manton (1958a) proposed, as a general rule for arthropods, that extension is performed hydraulically when the limb tip is off the ground but that a hydraulic mechanism could not account for the power strokes when the limb tip is on the ground. Parry and Brown, however, have made measurements of pressure which indicate that in spiders a hydraulic mechanism can account not only for the power strokes of walking (1959a) but also for the jump of salticid spiders (1959b). Because of the marked similarity in morphology between the hinge joints of *Limulus* and those of spiders, it has been assumed that the horseshoe crab also has a hydraulic mechanism of leg extension (see Pringle, 1956).

The average prosomal blood pressures of 14.1 mm Hg at rest to 23.8 mm Hg during activity found in *Limulus* are similar to the "typical" blood pressures found in most arthropods (Prosser and Brown, 1961; Inada, 1947). These pressures are much lower than the blood pressure of spiders with hydraulic leg extension. The blood pressure of *Tegenaria* ranges from 50 mm Hg at rest to about 400 mm Hg with activity (Parry and Brown, 1958a).

As seen in the results the average leg blood pressure in *Limulus* is 5.1 mm Hg for the flexed position of the femoro-patellar joint and 6.2 mm Hg for the extended position of the same joint; as mentioned before, this difference is not statistically significant. If a hydraulic mechanism of hinge joint extension were operating, a large and reliable difference in blood pressure would be expected when the extension occurred; namely, a difference in pressure which would be great enough to account for extension of the hinge joint. Not only is there no significant change in *in vivo* blood pressure in the leg on flexion and extension of the hinge joint, but, as can be seen in Figure 4, the blood pressure measured in the legs is far below the pressure needed to account for the amount of extension that occurs at the femoro-patellar joint in the living animal.

In spiders, bleeding or dehydration leads to loss of the ability to extend the hinge joints (Ellis, 1944). Further evidence supporting the conclusion that the extension of the hinge joints of *Limulus* is not effected hydraulically is the fact that all normal leg movements, including the maximum normal extension of the femoro-patellar joint, are observed in *Limulus* after massive blood loss. Although this observation still leaves open the possibility that in *Limulus* a local change in blood pressure could effect the extension, this seems unlikely since (1) pressure measurements in the legs were made near or at the hinge joint and (2) no structure or structures were found in the legs or near the legs that could isolate or pressurize a section of the leg or other compartment. Further, if a hole is drilled in the exoskeleton adjacent to the hinge joint at various sites, there is no impairment of extensor activity.

Haemocoelic hydraulic locomotory mechanisms probably evolved as an adaptation for burrowing in soft-bodied animals (Manton, 1961). However, with the acquisition of a stiff integument, most arthropods use direct muscular locomotory mechanisms rather than hydraulic systems. Notable exceptions occur in some or all spiders and perhaps in barnacles (Cannon, 1947) and some myriapods (Manton, 1958a), although direct evidence is lacking. In spite of the morphological similarity between the legs of *Limulus* and those of spiders, *Limulus,* according to the results of this study, has no hydraulic means of leg extension. At least partial hydraulic extension, however, occurs in spiders and may occur in other terrestrial arthropods with several hinge joints in the legs. Without hydraulic extension such animals might have difficulties in supporting their body weights, as happens in spiders following bleeding or dehydration (Ellis, 1944).

I wish to thank Dr. S. A. Wainwright for his advice throughout the course of this work and my husband for his extensive help. I was supported in part by a grant from the Cocos Foundation.

SUMMARY

The femoro-patellar hinge joints of *Limulus* show considerable extension in spite of their lack of extensor muscles.

I propose a mechanical scheme for extension of these extensorless hinge joints. The extension is performed by the combined action of the depressors of the trochanter, the depressors of the femur, and, especially in the case of the powerful last pair of legs, the coxal remotor muscles. The femoro-patellar joint extends passively due to (1) the effect of these muscles, (2) the force of the weight of the leg when off the ground, and (3) the force exerted by the limb tip against the substratum when the leg is on the ground.

Measurements of hydrostatic pressure in *Limulus* indicate that a hydraulic mechanism is not an effector of femoro-patellar extension. There is no evidence that an elastic mechanism functions in extending this joint.

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OXYGEN CONSUMPTION AND RESPIRATORY ENERGETICS IN THE SPINY LOBSTER, *PANULIRUS INTERRUPTUS* (RANDALL)

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This paper is part of a series designed to examine trophic and energy relationships in large decapod crustaceans. Information about metabolic rates, generally determined by measuring oxygen consumption (QO_2), and metabolic regulatory mechanisms is of basic importance in defining the metabolic energy budget (Paine, 1965) of an animal. Metabolic rates determine the amount of energy expended in producing biomass and therefore are also of importance in determining the role of the animal in community metabolism. Little information of this nature has been published on palinurids, and none on the California spiny lobster, *Panulirus interrupts,* an exemplary decapod.

In general, animals are either oxygen regulators or conformers according to the dependency of their rate of oxygen consumption on oxygen concentration (pO_2) in the environment. Regulating animals maintain a fairly constant rate of metabolism throughout a range of O_2 concentration. At some critical pO_2 (P_c) less than saturation, these animals will cease to regulate and will become dependent on the external pO_2. Most aquatic vertebrates, all known terrestrial vertebrates, and probably all terrestrial insects are regulators (Prosser and Brown, 1961). Conformers are animals whose metabolism is entirely dependent on the environmental pO_2. Fewer animal groups exhibit this mechanism. Most parasitic worms plus some crustaceans are conformers (Wolvekamp and Waterman, 1960; Prosser and Brown, 1961).

The dependency of QO_2 on body weight is a well-documented phenomenon in the animal kingdom and seems to be most evident in animals weighing between 1 and 1000 g (Edwards, 1946; Zeuthen, 1953; Wolvekamp and Waterman, 1960; Prosser and Brown, 1961). Most specimens of postpuerulus *P. interruptus* sighted or sampled by the author near San Diego, California are included within this weight range. It is also well-documented that, in poikilotherms, metabolism generally varies directly with the environmental temperature (Prosser and Brown, 1961).

In this study weight-specific respiration rates and the effects of environmental oxygen concentration, body weight, environmental temperature, and level of animal activity on these rates were determined for *P. interruptus* as a measure of metabolic energy lost under various conditions. The rates of energy expended in the process of active and resting metabolism, expressed as cal/g/day, were derived from these data by appropriate conversions.

MATERIALS AND METHODS

Specimens of *P. interruptus* (an eastern North Pacific palinurid of commercial importance)used in this study measured 4–9 cm from the eyes to the posterior end of the cephalothorax (carapace length). This size range included the majority of lobsters found near San Diego, California, where the sample population was obtained during the spring of 1967. Animals to be used in respiration measurements were held in 75-liter aquariums at 16° C and fed on the flesh of the California mussel, *Mytilus californianus*. Only those lobsters that fed in the laboratory were used.

Respiration rates of individual *P. interruptus* were measured by using a 24.0 × 9.4 × 15.1 cm (15 liter) rectangular plexiglass chamber fitted with a respiration head which was sealed by two rubber "O" rings in an 18.3 cm diameter opening in the top of the chamber. The head had an opening for insertion of a Yellow Springs BOD type polarographic oxygen electrode (Kanwisher, 1959) and two plexiglass pipes which were connected to a pump for circulating water through the respiration chamber. The exhaust pipe was directed downward for maximum circulation of water within the chamber; the intake pipe was directed horizontally and ended just under and to the side of the electrode membrane, in order to draw water across it. The chamber had a small hole fitted with a rubber stopper for insertion of a thermometer and for removal of water when the respirometer was not in operation.

In order to facilitate the removal of trapped air from the system, the respirometer was submerged in a plexiglass aquarium filled with sea water. The aquarium and water circulating tubing were placed in a 190 liter (50 gal) water bath. Another larger hose from the exhaust of the water bath pump was coiled around the respirometer inside the aquarium in order to increase cooling efficiency. All respiration determinations were made with the cover of the water bath closed. The entire system allowed the temperature of the sea water inside the respirometer to to be maintained within ±0.3° C. This procedure also maintained the animal in darkness and free from external laboratory disturbances during the measurements.

The oxygen electrode was connected through a Yellow Springs Instrument bridge circuit to a variable span stripchart potentiometer recorder, which allowed continuous monitoring of oxygen concentration in the respirometer. The polarographic electrode system was calibrated for air saturation level of oxygen concentration by placing an air stone and the electrode in a beaker of sea water at the experimental temperature for 5–10 min and adjusting the bridge circuit.

The respirometer system was filled with natural sea water filtered through coarse, crepe-surface filter paper and adjusted to the experimental temperature. Preceding each respiration determination, a control run of approximately one hour was made without the lobster in the chamber to determine the effect of bacterial respiration and possible unknown effects on the potentiometer recordings. This was followed by a 2–3 hour run with the lobster in the chamber. After each run the wet weight of the animal was recorded after vigorously shaking the animal to remove excess water. The temperature of the water in the respirometer was determined at the beginning and end of the control and experimental runs. The water in the respirometer was changed before each run and in the aquarium containing the respirometer every third run, for occasionally there was some exchange of water due to handling between the respiration chamber and the aquarium before and after each run.

Experimental animals were placed without food in a second plexiglass aquarium in the water bath to allow them to acclimate to the experimental conditions and temperature for 24 hours prior to placing them in the respirometer. Twenty-four hours was considered sufficient time for the animals to adjust to the small differences between the temperature of the holding tanks and the experimental temperatures. Respiration measurements were made on 10 lobsters at 13° C, 12 lobsters at 16° C, and nine lobsters at 20° C. These temperatures encompass the normal temperature range to which this species is subjected in nature.

In order to examine the effect of pO_2 on weight-specific oxygen consumption (QO_2), respiration rates were determined separately within the following pO_2 ranges for all respiration records:

pO_2 Range	Mean pO_2
6.5–6.0	6.25
6.0–5.5	5.75
5.5–5.0	5.25
5.0–4.5	4.75
4.5–4.0	4.25
4.0–3.5	3.75
3.5–3.0	3.25
3.0–2.5	2.75
2.5–2.0	2.25
2.0–1.5	1.75
1.5–1.0	1.25
1.0–0.5	0.75

The change in oxygen concentration (ppm/hr) was determined by dividing each 0.5 ppm increment by the time period between the limits of the increment of a given pO_2 range. The respiration chamber, hoses, and pump contained a total water volume of 15.42 liters. The change in oxygen concentration/hr was converted to oxygen consumption in ml/hr, based on the volume of the respirometer minus the volume of the lobster and the relationship: 1 ppm = 0.698 ml O_2/1 water. The volumes of representative lobsters were measured by water displacement and are given below:

Carapace length (cm)	Vol (liter)
5	0.08
6	0.16
7	0.21
8	0.43
9	0.63

The resultant oxygen consumption value was divided by the weight of the animal to obtain the weight-specific oxygen consumption in ml/g/hr for each pO_2 range.

In order to examine the effect of pO_2 on QO_2, standard linear and curvilinear regression analyses (Steel and Torrie, 1960) were made on these variables. Analyses of variance (Steel and Torrie, 1960) were used to test for the significance of the linear and curvilinear regressions. Analyses of variance were also used to test for the significance of curvilinearity, that is, whether the use of the 2nd degree polynomial reduced the variance about the regression line significantly more than did the linear regression.

The QO_2 is, of course, highly influenced by activity. Resting metabolism is distinguished from basal metabolism, which is difficult to determine in any animal except man. The QO_2 of *P. interruptus,* measured while the animal remained motionless and relaxed for a period of an hour or more, was assumed to represent resting metabolism. The lobsters were generally excited and moved about actively when placed in the respirometer. Inspection of the slopes of the respiration records suggests that the animals required approximately 30 minutes to settle down to a uniform low level of metabolic activity. It was assumed that the level of activity exhibited by individuals during the initial 30 minute period was representative of their peak level of activity in nature while foraging for food. The remainder of the run was used to determine resting metabolism.

P. interruptus remains fairly passive during the daylight hours, but begins foraging for food at sundown and continues until sunrise (Winget, unpublished). Therefore, the daily oxygen consumption is a combination of resting and active rates. One method of determining the mean daily rate would be to assume that half of the 24-hour period is spent in a state of active metabolism and half in a state of resting metabolism. This assumes, however, that the animal is continually active throughout the 12-hour period of darkness and is probably an overestimation.

An adaptation of a model proposed by McNab (1963) for the energy budget of a mouse gives a more accurate and conservative estimate of the daily oxygen consumption. This estimate is expressed in the equation:

$$M = f(T_c) + g(t)$$

where

M = metabolism
$f(T_c)$ = the passive rate as a function of time at a constant temperature,
$g(t) = \frac{1}{2}M_a[\cos(kt) + |\cos(kt)|]$ and expresses the increment of O_2 consumption due to activity as a function of time,
M_a = the increment at peak activity, and
$\cos(kt) + |\cos(kt)|$ = an expression of periodicity.

This model divides a period, such as 24 hours, into two parts: one where $g(t) = 0$, and one where $g(t)$ is greater than 0. The constant k aligns the x axis along the desired time scale. For this paper, $k = \pi/12$. At t_0 (midnight), $\cos(kt) = 1$ and $g(t) = M_a$. From t_6 to t_{18} $\cos(kt)$ is negative and $g(t) = 0$. At t_{24}, $g(t) = M_a$ again. To determine the total oxygen consumption over a 24-hour period, M is used as a derivative with respect to t and integrated from $t = 0$ to $t = 24$.

Results and Discussion

Effect of environmental oxygen concentration

Weight-specific oxygen consumption during resting metabolism and the effect of pO_2 on QO_2 at 13°, 16°, and 20° C for 31 specimens of *P. interruptus* are given in Table I. The mean of each pO_2 range was used as the independent variable in the regression analyses described in the Materials and Methods section. These variables were divided into groups and considered separately for each temperature as follows:

13° C pO_2(ppm)	16° C pO_2(ppm)	20° C pO_2(ppm)
6.50–4.75	5.25–2.75	4.75–2.75
4.25–3.25	2.25–0.75	2.25–0.75
6.50–3.25		
2.25–1.25		

In order to reduce the influence of differences in body weight on the regressions, the QO_2's corresponding to lobster weights of 0–199, 200–299, 300–399, 400–499, and 500–599 g were analyzed as separate sets of dependent variables. The variables used in each individual analysis are separated by lines in Table I. Only those groups of data which contain four or more QO_2 values were used.

TABLE I

Weight-specific oxygen consumption in ml/g/hr during resting metabolism of P. interruptus

Wet body weight (g)	ET* °C	QO_2 during resting metabolism at pO_2 levels between 0.5–6.5 ppm											
		6.5–6.0 $\bar{X}$ 6.25	6.0–5.5 5.75	5.5–5.0 5.25	5.0–4.5 4.75	4.5–4.0 4.25	4.0–3.5 3.75	3.5–3.0 3.25	3.0–2.5 2.75	2.5–2.0 2.25	2.0–1.5 1.75	1.5–1.0 1.25	1.0–0.5 0.75
56.0	13	0.0766											
100.8			0.0638	0.0532									
214.0			0.0498	0.0498	0.0498								
233.8		0.0330	0.0281	0.0281									
243.5						0.0437	0.0375	0.0375					
279.4				0.0207	0.0207								
293.9					0.0310	0.0361	0.0361	0.0361					
328.4			0.0388	0.0277	0.0242	0.0277							
342.7						0.0420	0.0265	0.0265	0.0327	0.0265			
393.6					0.0367	0.0367	0.0367	0.0367	0.0287	0.0234	0.0196	0.0128	
216.4	16					0.0509	0.0360						
245.0						0.0589	0.0589	0.0498	0.0498	0.0366	0.0281		
250.0							0.0539	0.0326	0.0265	0.0219	0.0219	0.0132	
202.0								0.0470	0.0572	0.0290	0.0290	0.0170	
335.4					0.0476	0.0633	0.0633	0.0476	0.0476	0.0476	0.0317		
354.5						0.0476	0.0476	0.0476	0.0327	0.0327	0.0236	0.0133	0.0077
364.5								0.0627	0.0509	0.0364	0.0364	0.0176	0.0120
366.6								0.0578	0.0462	0.0462	0.0230	0.0172	0.0076
420.7				0.0497	0.0497	0.0373	0.0497	0.0373	0.0373	0.0373	0.0373		
429.9					0.0582	0.0488	0.0417	0.0488	0.0488	0.0292	0.0243		
524.3								0.0589	0.0394	0.0394	0.0296	0.0236	
578.7								0.0429	0.0429	0.0357	0.0268	0.0214	0.0089
124.3	20				0.0938	0.0938	0.0766	0.0644					
155.5						0.1290	0.0738	0.0738	0.0738	0.0502			
171.0							0.0658	0.0658	0.0658	0.0533	0.0378		
204.7									0.0629	0.0520	0.0258	0.0184	
227.1							0.0533	0.0845	0.0612	0.0612	0.0470	0.0377	
255.9								0.0758	0.0758	0.0758	0.0425	0.0425	0.0177
372.6							0.0841	0.0561	0.0561	0.0561	0.0422	0.0210	
379.4									0.0579	0.0579	0.0388	0.0330	0.0192
387.9							0.0648	0.0648	0.0648	0.0462	0.0270	0.0231	

* Environmental temperature.

In those ranges where the mean pO_2 was 2.25 ppm or less, four out of seven curvilinear and five out of seven linear regressions were significant ($p < 0.05$). In no case was curvilinearity significant ($p > 0.05$). In those ranges where the mean pO_2 was 2.75 ppm or greater, one out of 13 linear regressions was significant ($p < 0.05$). The single significant regression was for variables corresponding to lobsters at 20° C which weighted less than 200 g.

Regulation of oxygen consumption

The results of the analyses indicate that, in general, the QO_2 of *P. interruptus* is not affected by oxygen concentration at pO_2 levels above 2.5 ppm, but that QO_2 declines at pO_2 values less than 2.5 ppm. The conclusion of this study is that *P. interruptus* is a regulator and that P_c is approximately 2.5 ppm. More extensive analyses, incorporating larger sample sizes and regression analyses of data above and below pO_2 values close to 2.5 ppm are needed to depict more accurately the range of pO_2 values within which P_c falls. However, examination of the increasing or decreasing trends of the individual QO_2 values in Table I suggests that P_c lies between 2 and 3 ppm, or 25–40% air saturation. The P_c for *P. interruptus* is similar to those of two crayfish, *Orconectes* (20–40% air saturation) and *Astacus* (20% air saturation), and other regulatory crustaceans reported by Weymouth, Crismon, Hall, Belding, and Field (1944); Wolvekamp and Waterman (1960); and Prosser and Brown (1961).

If the QO_2–pO_2 relationships of *P. interruptus* are representative of *Panulirus* and related genera, this aspect of metabolism represents a distinctive physiological contrast between spiny lobsters (family Palinuridae) and lobsters of the family Homaridae. Thomas (1954) indicated that the QO_2 of *Homarus vulgaris* varies directly with the oxygen concentration of the water. It has also been shown that *H. americanus* (Amberson, Mayerson, and Scott, 1924) and *H. gammarus* (Wolvekamp and Waterman, 1960) are conformers, as are the crayfish, *Cambarus* (Maloeuf, 1936), the crab *Callinectes* (Wolvekamp and Waterman, 1960) and the horseshoe crab, *Limulus* (Prosser and Brown, 1961).

The mechanisms through which the regulation of oxygen utilization in crustaceans is effected are mainly circulatory and ventilation adaptations to changes in various environmental parameters (Prosser and Brown, 1961). Redmond (1955) found that the blood of *P. interruptus* reaches 95% saturation when the partial pressure of oxygen is about 15 mm Hg (9.4% air saturation) at 10° C, 25 mm at 15° C, and 30 mm at 20° C. The half saturation points (P_{50}), a more accurately determined figure for oxygen affinity, are 4, 6.5, and 9 mm, respectively. Redmond has determined, however, that even in highly oxygenated water, the immediate post branchial blood in *P. interruptus* is only about 50% saturated. This phenomenon indicates that oxygen diffusion across the gill surfaces is very slow and that the pO_2 may drop from near saturation to less than 10 mm Hg (6.3% air saturation). The finely branched gills are distributed over the entire lateral surface of the carapace and present a tremendous surface for exchange of O_2 and CO_2 with the ventilated water. This large gill area undoubtedly compensates for the low diffusion gradient. The lack of blood saturation, even under highly oxygenated environmental conditions, would indicate that QO_2 regulation is probably not affected appreciably by differential uptake of O_2 by the blood. To my knowledge, it is not known whether changes in pO_2 alter the heartbeat.

No specific information is available on the ventilation rates of *P. interruptus.* However, it is suspected that regulation of QO_2 may be through ventilation control. The ventilation rate of the oxygen conformer, *Homarus,* does not change under low pO_2's (Thomas, 1954). Fox and Johnson (1934) found that the crayfish, *Astacus fluviatilis,* regulates at least partially through ventilation control. When the pO_2 was lowered from 8.3 to 2.2 ml O_2/1, the scaphnognathite beat increased from 34 to 140 beats/min in this species.

The relationship between QO_2 and environmental oxygen supply has been discussed extensively in the literature. For many marine animals, including *P. interrupts,* this is probably a purely academic consideration, for they rarely exist under conditions of low oxygen supply. Serial dissolved oxygen measurements obtained at the U. S. Naval Electronics Laboratory Tower off Mission Beach, California (Ramsey, 1962) indicate that pO_2's from the surface to the bottom at a depth of 20 meters deviate little from air saturation levels. *P. interruptus* is usually found in similar areas of shallow, well-circulated water where the pO_2 is very close to saturation at all times. The analyses of the QO_2–pO_2 relationships of *P. interruptus* was undertaken to compare the dependency of the QO_2 of this species on oxygen concentration with this relationship of other crustaceans and to attempt to apply a method of determining meaningful QO_2 values which were measured over a wide range of oxygen concentrations, a problem which represents one of the major shortcomings of the type of respirometer system used in this study.

Weight-specific respiration rates

Because the regression analyses indicate that, in general, the QO_2 does not change significantly when measured from a mean pO_2 of 2.75 ppm (range 2.5–3.0 ppm) up to the highest pO_2 used in the experiments, the QO_2 for each lobster was determined as an average of those values measured at pO_2's higher than 2.5 ppm. These QO_2's are given in Table II.

TABLE II

Weight-specific oxygen uptake in P. interruptus *during resting metabolism in ml/g/hr*

Wet body weight (g)	Mean QO_2 from 6.5–2.5 ppm	Mean temperature of experiment (°C)	Wet body weight (g)	Mean QO_2 from 5.5–2.5 ppm	Mean temperature of experiment (°C)	Wet body weight (g)	Mean QO_2 from 5.0–2.5 ppm	Mean temperature of experiment (°C)
56.0	0.0766	12.8	216.4	0.0435	16.0	124.3	0.0822	19.8
100.8	0.0585	12.9	245.0	0.0544	15.9	155.5	0.0876	19.9
214.0	0.0498	12.5	250.0	0.0377	15.9	171.0	0.0658	19.9
233.8	0.0297	13.3	302.0	0.0521	16.0	204.7	0.0629	19.8
243.5	0.0396	12.5	335.4	0.0539	16.1	227.1	0.0663	19.8
279.4	0.0207	12.9	354.5	0.0439	15.9	255.9	0.0758	19.8
293.9	0.0348	13.0	364.5	0.0568	15.9	372.6	0.0654	19.6
328.4	0.0296	12.6	366.6	0.0521	15.5	379.4	0.0578	19.7
342.7	0.0319	13.0	420.7	0.0435	16.0	387.9	0.0648	19.8
393.6	0.0351	12.6	429.9	0.0492	16.0			
			524.3	0.0492	16.0			
			578.7	0.0429	15.9			

In order to examine the effect of body weight on QO_2, standard linear and curvilinear regression analyses (Steel and Torrie, 1960) were made on these variables. The significance of the regressions was tested with analysis of variance (Steel and Torrie, 1960). These regression analyses of the QO_2's in Table II on body weight and tests of significance were repeated with the data for individuals weighing less than 200 g deleted, in order to examine the influence of these smaller individuals on the regressions.

The results indicate that the QO_2 for *P. interruptus* weighing from 200–600 g is not significantly ($p > 0.05$) affected by body weight. At 13° C significant ($p < 0.05$) linear and curvilinear regressions appear when data for individuals weighing less than 200 g are included (curvilinearity significant, $p < 0.05$). QO_2's for individuals weighing less than 200 g were not measured at 16° C. More data are needed to determine the effect of body weight on QO_2 for individuals weighing less than 200 g. However, it is suspected, based on the available data, that the effect is significant.

Thomas (1954) presented a graph of the regression of QO_2 on body weight in *Homarus vulgaris.* The standard linear regression and analysis of variance test of the significance of the regression (Steel and Torrie, 1960) were run by the author on values for weight and QO_2 estimated from Thomas's graph. The regression was significant ($p < 0.05$) and indicates that in *H. vulgaris,* QO_2 is dependent on body weight. This dependency may illustrate another physiological contrast between the Palinuridae and Homaridae. If *P. interruptus* is representative of Palinuridae metabolism, the QO_2 of this group of animals may be independent of body weight within the 200–600 g range.

TABLE III

Oxygen consumption in ml/g/hr in decapod crustaceans

Species	Wet body weight (g)	Temperature (°C)	QO_2 (ml/g/hr)	Reference
Macrura				
Scyllaridae				
Palinurus elephas	—	15	0.044	Wolvekamp and Waterman (1960)
Panulirus interruptus	200–600	13	0.034	This study
		15	0.048	
		16	0.048	
		20	0.066	
P. argus	300	30	0.091**	Maynard (1960)
Nephropsidae				
Homarus americanus	189	15	0.035	Thomas (1954)
	230	22	0.037	
	324	22	0.039	
H. vulgaris	400*	15	0.063*	Thomas (1954)
	680*	15	0.040*	
H. gammarus	—	15	0.068	Wolvekamp and Waterman (1960)
Orconectes immunis	—	25	0.160–0.170	Wolvekamp and Waterman (1960)
Brachyura				
Carcinus maenas	—	16	0.052–0.071	Wolvekamp and Waterman (1960)
Cancer pagurus	—	16	0.107	
Ocypode quadrata	—	26	0.196	
Pugettia producta	—	15	0.032–0.170	
Mean of 54 crustaceans	—	15	0.108	Wolvekamp and Waterman (1960)

* Values approximated from graphs (Thomas, 1954).

** Calculated from formula $QO_2 = 0.24W^{-0.17}$ (Maynard, 1960).

In the absence of significant regression ($p > 0.05$) for animals larger than 200 g, mean QO_2's corresponding to these animals were used to predict the oxygen consumption of *P. interruptus.* At 13°, 16°, and 20° C, the QO_2 is 0.0339, 0.0483, and 0.0655 ml/g/hr, respectively. These values are compared with QO_2's of other decapod crustaceans in Table III. At 15° and 16° C, the QO_2 of *P. interruptus* is similar to that of another palinurid, *Palinuris elephas.* From the data in Table III, QO_2's for the palinurids appear to fall within the range of values for homarids, but are generally a little lower than the astacid, *Orconectes,* and the Brachyura.

The relationship between oxygen consumption and body weight is often expressed by the equation:

$$\log O_2 = \log a + b \log W$$

or

$$O_2 = aW^b,$$

when

a = the y intercept,
b = the regression slope, and
W = the weight.

When determining values for this equation, oxygen is measured as total O_2 consumption of the animal/hr and not on a weight-specific basis.

Whole animal oxygen consumption values for *P. interruptus* were determined by multiplying the QO_2's in Table II by the corresponding body weights of the experimental animals. Analyses were made of the regressions of these nonweight-specific O_2 consumption values on animal body weight in logarithmic form. The y intercept and slope of the regression line at each temperature are presented below:

a		b	
Log	Non-log		Temperature (°C)
−0.2120	0.614	0.483	13
−1.1447	0.072	0.935	16
−0.5972	0.253	0.763	20

Although the b values vary considerably, their average is 0.73.

Zeuthen (1953) made a similar analysis of large poikilotherms, including crustaceans, amphibians, fish, and reptiles, and obtained an average slope of 0.76, a very similar value to the slope obtained in this study for *P. interruptus.* Zeuthen (1953) states that the QO_2 of homeotherms varies with a power of about 0.75 (b value) of the body weight. Weymouth *et al.* (1944) reported that the QO_2 of the kelp crab, *Pugettia producta,* is inversely proportional to the size of the animal ($b = 0.788$). They also plotted the log O_2 and log weight of various Crustacea from 27 mg to 520 g with a resultant composite slope of 0.826. Paine (1965) obtained a b value of 0.885 for the opisthobranch, *Navanax inermis,* and Richman (1958) obtained a slope of 0.881 for *Daphnia pulex.* Thus it appears that the slope of oxygen consumption plotted against weight on double logarithmic paper for *P. interruptus* is close to poikilotherms in general but a little lower than many other crustaceans.

Effect of environmental temperature

Standard regression analysis and analysis of variance test of significance (Steel and Torrie, 1960) of the QO_2's on mean temperatures for all body weights in Table II show a significant linear regression ($p < 0.05$). The linear equation is:

$$Y = 0.00418X - 0.0150$$

Predictions of QO_2's at temperatures other than those measured may also be obtained with the Q_{10}, the factor by which chemical and physical reactions are accelerated due to a 10° C change in temperature, obtained from the formula:

$$Q_{10} = \left(\frac{K_1}{K_2}\right)^{10/(t_1-t_2)}$$

where in metabolic studies,

$K_1 =$ the QO_2 at t_1 (temperature, °C), and
$K_2 =$ the QO_2 at t_2.

Using QO_2 values of 0.0339, 0.0483, and 0.0655 for temperatures of 13°, 16°, and 20° C, respectively, the Q_{10} for *P. interruptus* in the temperature range of 13–16° C is 3.25, and for 16–20° C it is 2.14. These values are similar to Q_{10}'s for crustaceans and poikilotherms in general (Wolvekamp and Waterman, 1960; Prosser and Brown, 1961).

TABLE IV

Weight-specific oxygen uptake in P. interruptus *during active metabolism in ml/g/hr*

Wet body weight (g)	QO_2 at 13°C (ml/g/hr)	Wet body weight (g)	QO_2 at 16°C (ml/g/hr)	Wet body weight (g)	QO_2 at 20°C (ml/g/hr)
56.0	—	216.4	0.1001	124.3	0.1197
100.8	—	245.0	0.1044	155.3	—
214.0	0.0747	250.0	0.0750	171.0	0.0845
233.8	0.0618	302.0	0.0832	204.7	0.0778
243.5	0.0656	335.4	0.0792	227.1	0.0956
279.4	—	354.5	0.0776	255.9	0.0965
293.9	0.0723	364.5	0.0870	372.6	0.1196
328.4	0.0485	366.6	0.0827	379.4	0.0947
342.7	0.0649	420.7	0.0622	387.9	0.1058
393.6	0.0585	429.9	—		
		524.3	0.0886		
		578.7	0.0670		

Effect of animal activity

The QO_2 values determined during active metabolism, as described in the Materials and Methods section, are given in Table IV. Activity will obscure any relationship between QO_2 and weight or pO_2. The mean of the active QO_2 values at each temperature was therefore used as the best estimate. These are: 0.0638, 0.0825, and 0.0983 ml/g/hr at 13°, 16° and 20° C, respectively. These values along with the QO_2's for resting metabolism were used in the McNab model described in the Materials and Methods section to determine oxygen consumption over a 24-hour period.

At 13° C, the integral from this model is:

$$\int_0^{24}\left[.0339 + \frac{.0299}{2}\left(\cos\frac{\pi}{12}t + \left|\cos\frac{\pi}{12}t\right|\right)\right]dt$$

Using similar integrals for the other temperatures, daily oxygen consumptions were estimated to be 1.04, 1.42, and 1.82 ml/g at 13°, 16°, and 20° C, respectively. The active increment is similar for all three temperatures (0.229, 0.261, and 0.251 ml/g), indicating that it is not influenced appreciably by temperature.

Metabolic rates in caloric form

Vonk (1960) concludes from data reported by Renaud (1949) for the crab, *Cancer pagurus,* that the chief energy source in this species is the metabolism of glycogen and fatty acids and indicates that this is probably true for crustaceans in general. No data are available on the composition of *P. interruptus.* However, the composition of many decapod crustaceans is probably fairly similar. Therefore, data on the composition of *C. pagurus* were used. Vonk gives the per cent of fresh weight of glycogen and fatty acids in *C. pagurus* as 0.20 and 1.67, respectively. These are average values, for the composition of decapods varies with the molting cycle.

Brody (1964) lists the energy values liberated for fat and glycogen when burned with 1 ml of oxygen as 4.6 and 5.14 cal, respectively. The mean of these values, weighted by the per cent composition of each, or 4.66 cal/ml O_2 consumed, was used to determine the metabolic expenditure of energy in *P. interruptus.* This value is slightly less than 5.0 used by Paine (1965) for *Navanax inermis,* by Richman (1952) for *Daphnia pulex,* and 4.8 used by Golley and Gentry (1964) for the harvester ant, *Pogonomyrex badius.*

When the daily oxygen consumption is multiplied by 4.66, the mean energy utilized in metabolism by *P. interruptus* at 13°, 16°, and 20° C is 4.85, 6.62, and 8.48 cal/g/day, respectively, for individuals in the range of 200–600 g during the intermolt period.

CONCLUSIONS

The amount of energy expended by *P. interruptus* in converting ingested food to utilizable substances or in metabolizing reserve energy sources during the intermolt period is estimated to vary between 5 and 8 cal/g/day throughout the year. A comparison of QO_2 values for *P. interruptus* and other decapod crustaceans indicates that 5–8 cal/g/day is a rough estimate of daily metabolism in other palinurid and homarid lobsters and that a slightly higher value would apply in several crab species. However, knowledge of diurnal metabolism in these species is necessary to substantiate this conclusion. If it is assumed that the average weight of *P. interruptus* near San Diego, California is between 200 and 300 g, the daily metabolic energy loss is on the order of 1.0–1.5 kcal per individual during the winter and 1.5–2.5 kcal during the summer.

This paper only initiates a determination of the role of a large decapod in community metabolism. For a complete study, estimates are needed of metabolic energy lost during all stages of the molting cycle as well as knowledge of population structure and dynamics of the species involved.

I sincerely thank Richard Ford and William Sloan of San Diego State College for their assistance in this project.

SUMMARY

This paper is part of a series designed to examine the trophic and energy relationships of large decapod crustaceans. The oxygen consumption and daily energy loss due to respiration were measured in the California spiny lobster, *Panulirus interruptus.* At 13°, 16°, and 20° C, the QO_2 was 0.0339, 0.0483, and 0.0655 ml/g wet body weight/hr, respectively, for individuals weighing between 200 and 600 g. Within this weight range QO_2 was independent of body weight but was dependent on temperature, yielding a Q_{10} estimate of 3.25 from 13°–16° C and a corresponding coefficient of 2.14 from 16°–20° C. The QO_2 of this species is fairly similar to that of other palinurid and homarid lobsters and somewhat lower than those of several crab species. *P. interruptus* is an oxygen regulator, with P_c located between 25 and 45% air saturation.

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STUDIES ON MEMBRANE TRANSPORT
V. TRANSPORT OF LONG CHAIN FATTY ACIDS IN *HYMENOLEPIS DIMINUTA* (CESTODA)[1]

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Several workers have studied the incorporation of higher fatty acids into the lipids of *Hymenolepis diminuta*. Various ^{14}C-labeled fatty acids including palmitate, stearate, oleate, and linoleate were incorporated into triglycerides of *H. diminuta, in vitro* (Jacobsen and Fairbairn, 1967), while linoleic acid was shown to be deposited in lipid droplets within the medullary parenchyma of the same species (Lumsden and Harrington, 1966). More recently *H. diminuta* has been shown to absorb oleic and linoleic acids and monoolein when these fats were in the presence of sodium taurocholate (Bailey and Fairbairn, 1968).

There is evidence that *H. diminuta* is incapable of *de novo* synthesis of higher fatty acids and therefore must rely on exogenous sources, although the tapeworm can carry out chain lengthening of absorbed fatty acids (Jacobsen and Fairbairn, 1967).

Arme and Read (1968) have demonstrated that there is a membrane locus involved in the mediated transport of short chain fatty acids by *H. diminuta*. It was therefore thought to be of interest to investigate the uptake of higher fatty acids with particular reference to the initial velocity of this transport.

Materials and Methods

The methods used in the present study for the maintenance of *Hymenolepis diminuta* in the laboratory and the study of membrane transport *in vitro* were essentially those described by Read, Rothman, and Simmons (1963). The outline of these methods is as follows: Young male rats (Holtzman Rat Co., Madison, Wisconsin) were infected *per os.* with 32 cysticercoids of *H. diminuta*, obtained

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from infected *Tenebrio molitor.* Rats received a diet of Purina Laboratory Chow. At 10 days post-infection rats were killed and the intestines removed to Krebs-Ringer-tris solution (KRT) at pH 7.4. Worms were flushed from the intestines with KRT and washed several times; they were then randomized into groups of five. Each group of five worms was preincubated in 10 ml of KRT for 30 minutes at 37° C and was then transferred to 4 ml of incubation medium for 2 minutes. Following incubation, worms were washed twice in KRT, dried on Whatman No. 5 paper and transferred to 2 ml of 70% ethanol. Worm lipid was extracted in ethanol for 24 hrs, after which worms were removed to a drying oven at 90° C. Worm dry weight was recorded after drying for 24 hours. Aliquots (1 ml) of ethanol extracts were pipetted on aluminum planchets (1.25 in diameter) and 0.25 ml of a 10 m*M* sodium bicarbonate solution added. Radioactivity was determined with a Nuclear Chicago gas flow counter.

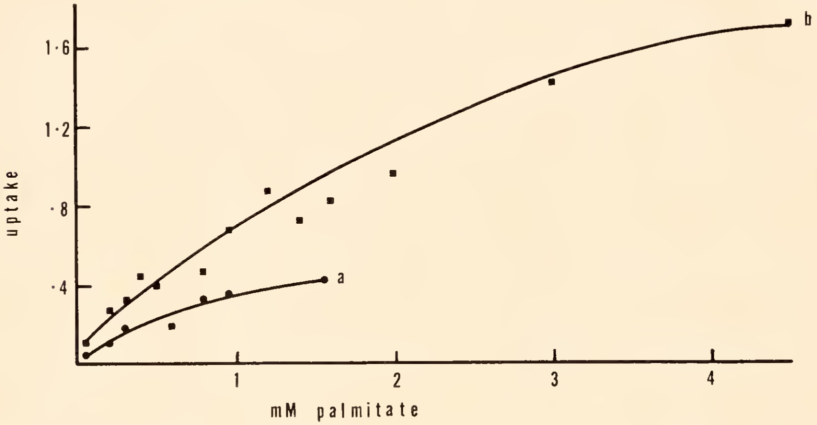


FIGURE 1. The uptake of ^{14}C-palmitate as a function of palmitate concentration. Curve a is uptake of palmitate diluted from a 2 m*M* stock solution and Curve b is uptake of palmitate diluted from a 10 m*M* stock solution. Each point is the mean of at least 8 replicates. Uptake is expressed in μMoles/g/2 min.

Unless otherwise stated, all incubation media contained sodium desoxycholate (1μ Mole/ml).

Radioactive palmitic acid, labeled with ^{14}C at the carbon-1 position, was obtained from Nuclear Chicago Corps. The specific activity was 38.5 μC/mg. Unlabeled sodium palmitate (A grade) was obtained from K & K Laboratories. "A" grade stearic, oleic, linoleic, and linolenic acids, and sodium taurocholate were obtained from Calbiochem. Chromatographically pure pentadecanoic, arachidic, and lignoceric acids were obtained from Sigma Chemical Co. and sodium desoxycholate, of enzyme grade purity, was purchased from Mann Research Laboratories. All other chemicals used were of reagent grade. The sodium salts of fatty acids were prepared by titration to pH 7.6–8.0 with sodium hydroxide.

Stock solutions of ^{14}C-palmitate (100 ml) were made up to contain either 0.5 μC/μMole(2m*M*) or 0.1 μC/μMole(10 m*M*). These solutions were diluted to the required final concentration with 2X KRT, bile salt, water, and other additions where required.

It is necessary to point out that the physical properties, particularly viscosity, of the incubation media varied according to the total concentration of fatty acid salt(s) present. For example, a medium that contained 0.025 μMoles of ^{14}C-palmitate/ml (the lowest concentration used) readily transmitted light whereas a solution that contained 4.5 μMoles of ^{14}C-palmitate/ml was completely opaque. Furthermore, when an additional fatty acid was included in the medium, the concentration of palmitate plus additional fatty acid required to produce definite opacity was lower than that of palmitate alone. No incubation medium was used that contained a flocculent precipitate. It is evident that the solubility of higher fatty acids is such that it imposes limitations on the concentration range over which the transport of long-chain fatty acids may be examined.

In the following account all uptake data are expressed in terms of μMoles ^{14}C-palmitate absorbed/g dry weight/2 min incubation.

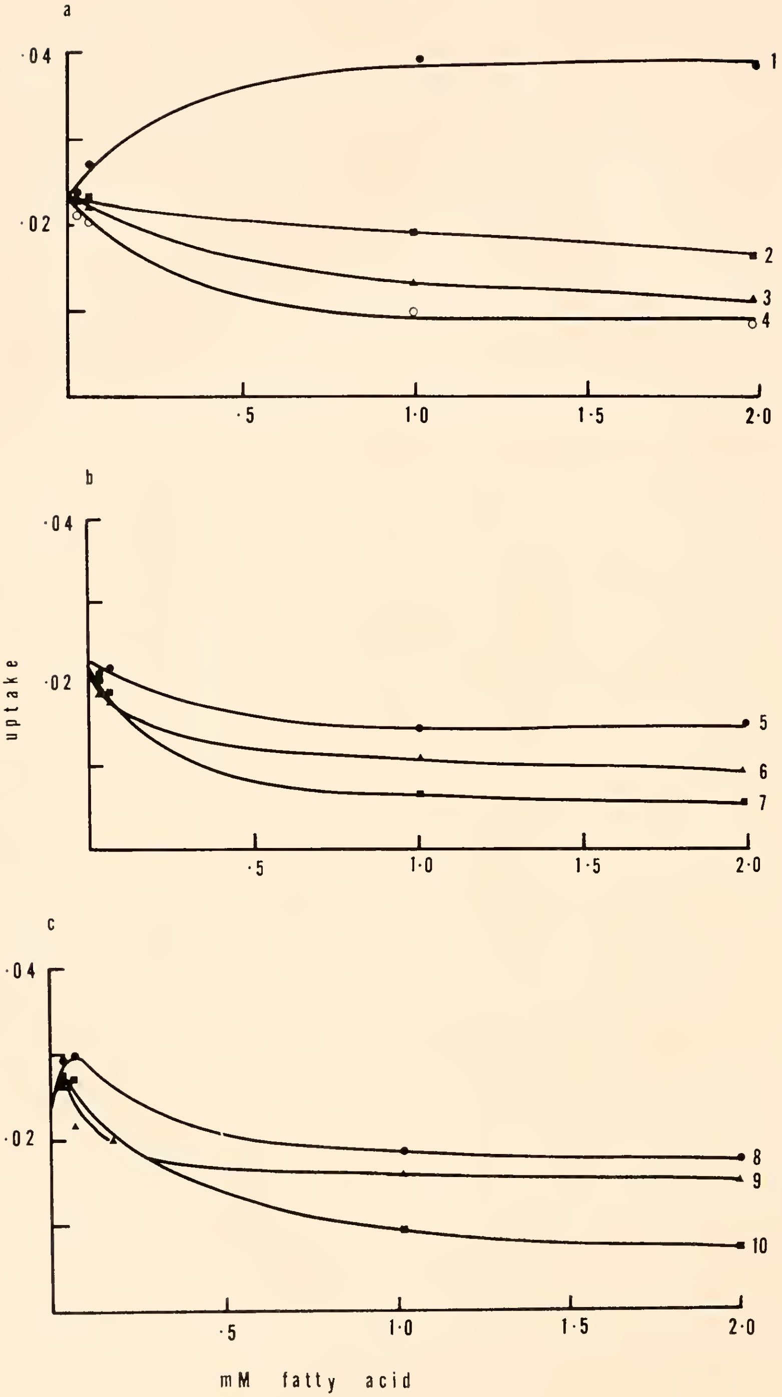
Results

Unless otherwise stated, the final concentration of ^{14}C-palmitate in the incubation media was achieved by dilution of a 2 m*M* stock solution.

Initial studies were carried out to determine the amount of labeled palmitate absorbed by *H. diminuta* during 2 min incubations. Figure 1 (curve a) shows that, over a concentration range of 0.025–1.55 m*M* palmitate, uptake was nonlinear and approached saturation. From these data it was possible, by plotting the reciprocal of uptake against the reciprocal of palmitate concentration (Lineweaver and Burk, 1934), to determine the transport constant, K_t (equivalent to Michaelis' constant, K_m), and the Vmax (extrapolated maximum velocity of a saturable system). K_t was calculated to be 0.71 m*M* and Vmax 0.48 μMoles/g/2 min.

Effects of fatty acids on palmitate transport

A wide variety of fatty acids were investigated for their effects on palmitate uptake by the tapeworm. In initial experiments palmitate concentration was kept constant (0.025 m*M*) while the concentration of the additional fatty acid was increased from parity with palmitate to its upper limiting physical concentration (*i.e.*, the concentration at which precipitation occurred). The results of experiments using long chain fatty acids are shown in Figure 2. Effects on palmitate uptake were separable into three categories: Group I (Fig. 2a) stimulated uptake, the degree of stimulation being proportional to the concentration of additional fatty acid. Only laurate (12:0) produced an effect of this type. Group II (Fig. 2a, b) comprised those saturated fatty acids [myristate (14:0), pentadecanoate (15:0), heptadecanoate (17:0), stearate (18:0), arachidate (20:0), and lignocerate (24:0)] that inhibited palmitate uptake over their entire concentration range. In no case was the uptake of palmitate fully inhibited; the maximum per cent inhibition varied with the fatty acid present. Group III (Fig. 2c) contained unsaturated fatty acids [oleate (18:1), linoleate (18:2), and linolenate (18:3)]. At concentrations approximately equal to the concentration of palmitate, these fatty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uptake
5
6
7
·5
1·0
1·5
2·0
c
·04
·02
8
9
10
·5
1·0
1·5
2·0
mM fatty acid

TABLE I

Effects of sodium salts of fatty acids on ^{14}C-Palmitate uptake

Palmitate concentration	Addition	Ratio addition: Palmitate	Uptake ± standard error µMoles/g/2 min.	% Effect	N	P	K_i	Effect
0.1 mM	None	—	0.074 ± 0.005	—	16	—	—	—
0.1 mM	Formate	40:1	0.070 ± 0.004	5.4	8	>0.6	—	None
0.1 mM	Acetate	40:1	0.069 ± 0.004	6.3	8	>0.5	—	None
0.1 mM	Propionate	40:1	0.074 ± 0.004	0.7	8	>0.9	—	None
0.1 mM	Butyrate	40:1	0.078 ± 0.005	6.0	8	>0.6	—	None
0.025 mM	None	—	0.022 ± 0.002	—	80	—	—	—
0.025 mM	Decanoate	40:1	0.024 ± 0.000	8.2	8	>0.1	—	None
0.025 mM	Laurate	40:1	0.039 ± 0.002	75.6	8	<0.001	—	Stimulation†
0.025 mM	Myristate	40:1	0.019 ± 0.001	15.5	8	<0.01	—	Inhibition†
0.025 mM	Pentadecanoate	40:1	0.013 ± 0.000	41.6	8	<0.001	4.9	Inhibition†
0.025 mM	Heptadecanoate	40:1	0.009 ± 0.000	57.5	8	<0.01	2.3	Inhibition†
0.025 mM	Stearate	40:1	0.011 ± 0.000	49.1	8	<0.01	2.3	Inhibition†
0.025 mM	Arachidate	40:1	0.014 ± 0.000	37.2	8	<0.001	3.5	Inhibition†
0.025 mM	Lignocerate	40:1	0.007 ± 0.000	67.3	8	<0.001	—	Inhibition†
0.025 mM	Oleate	1:1	0.029 ± 0.001	31.4	8	<0.001	4.5	Stimulation†
0.025 mM	Oleate	40:1	0.018 ± 0.000	17.2	8	<0.01	4.5	Inhibition†
0.025 mM	Linoleate	1:1	0.028 ± 0.002	25.2	8	<0.01	3.8	Stimulation†
0.025 mM	Linoleate	40:1	0.015 ± 0.002	33.2	8	<0.001	3.8	Inhibition†
0.025 mM	Linolenate	1:1	0.028 ± 0.001	27.9	8	<0.001	5.6	Stimulation†
0.025 mM	Linolenate	40:1	0.008 ± 0.000	62.8	8	<0.001	5.6	Inhibition†
0.025 mM	Palmitoleate	1:1	0.022 ± 0.001	1.3	8	>0.8	—	None

† Considered to be significant effects by application of Student's t test.

acids stimulated the uptake of palmitate. Further increase of fatty acid in the medium brought about inhibition of palmitate uptake.

Table I shows the uptake of palmitate (0.025 mM) in the presence of a number of fatty acids (1.0 mM); also included are the uptake data when unsaturated fatty acids were present at 0.025 mM. These data indicate the specificity of the palmitate transport site. Short chain fatty acids, formate through decanoate, had no effect on palmitate uptake, laurate had a pronounced stimulatory effect, while higher fatty acids showed varying degrees of inhibitory (and, in three cases, stimulatory) effect. The only higher fatty acid which was without effect on palmitate transport was palmitoleate ($CH_3(CH_2)_5CH{:}CH_2)_7-COOH$).
was more meaningful, however, to use a K_t and Vmax determined within each

To determine the nature of the inhibition of palmitate uptake by higher fatty acids, experiments were carried out in which the inhibitor concentration was maintained at 1.5 mM (a concentration above which no further inhibition occurred) and palmitate concentration was increased from 0.025 mM to 0.3 mM. Analysis of these data by the method of Lineweaver and Burk (1934) indicated that inhibitions by Group II and III fatty acids were competitive in nature. Because of

FIGURE 2. The effects of increasing concentrations of various fatty acids on the uptake of palmitate (0.025 mM). 1: laurate, 2: myristate, 3: pentadecanoate, 4: heptadecanoate, 5: arachidate, 6: stearate, 7: lignocerate, 8: oleate, 9: linoleate, 10: linolenate. Each point is the mean of 8 replicates.

their apparent insolubility, inhibitions with myristate and lignocerate could not be analyzed in this way. Using the previously determined K_t and Vmax for the uninhibited uptake of palmitate, it was possible to calculate the inhibitor constants for these fatty acids applying the equation given by Arme and Read (1968); it experimental group. The K_i for each inhibitory fatty acid is given in Table I. It will be noted that, while linolenate inhibited palmitate uptake by more than 60% its K_i was 5.6, an unexpectedly high value. (The smaller the K_i, the greater the inhibitor affinity for the transport site and hence the greater the per cent inhibition.) An explanation for this apparent discrepancy will become evident when the diffusion component of palmitate uptake is discussed below. Inhibitor constant, K_i, could not be determined for lignocerate due to the formation of a precipitate at higher concentrations. However, by applying the method of Dixon (1953), all inhibitory fatty acids, with the exception of oleate, linoleate, and linolenate, were shown to be partially competitive inhibitors of palmitate transport.

Effects of compounds other than fatty acids on palmitate uptake

To ascertain the degree of specificity of the palmitate transport site, a variety of biologically important substances were examined for their effects on palmitate transport. In these experiments palmitate concentration was maintained at 0.1 m*M* while the test substances were kept at a concentration of 4.0 m*M*. None of the following inhibited palmitate uptake: succinate, malate, glutamate, aspartate, citrate, adenine, uracil, betaine, ouabain, dextrose, galactose, leucine, alanine, phenylalanine, sarcosine, lysine and 2,4-dinitrophenol (at 2.5 m*M*).

A diffusion component in palmitate uptake

The uninhibited uptake of palmitate (Fig. 1a) showed non-linearity over the concentration range tested, and it was therefore impossible to determine the significance of diffusion of palmitate through the tegument in this system. However, by reference to Figure 2a, b, and c, it is evident that no inhibitor completely precluded uptake of palmitate. It is, therefore, apparent that there are at least two systems for the entry of palmitate; the first is a transport that can be inhibited to a varying extent by fatty acids, but is unaffected by other groups of compounds, and a second, which is not affected by fatty acids, and may be diffusion. It was possible to investigate this second system further in the following manner: Using heptadecanoate, lignocerate, and linolenate at concentrations above which no further inhibition of palmitate uptake occurred [but varying the absolute concentrations of both palmitate (0.0125 m*M*, 0.025 m*M*, and 0.05 m*M*) and inhibitor (0.25 m*M*, 0.5 m*M*, and 1.0 m*M*) whilst maintaining a constant 20:1 inhibitor to substrate ratio] it was possible to show that palmitate uptake, after inhibition, was linear with respect to its concentration. This relationship indicates first, that residual palmitate uptake following inhibition was diffusion, although it is also possible that the data from these experiments suggest the presence of a second mediated uptake system, saturable at concentrations in excess of those used; and second, that the high per cent inhibitions by lignocerate and linolenate were not competitive beyond the maximum per cent inhibition with heptadecanoate (57.5%). At high concentrations both lignocerate and linolenate produced very viscous media and it seems likely that non-specific interference with palmitate uptake

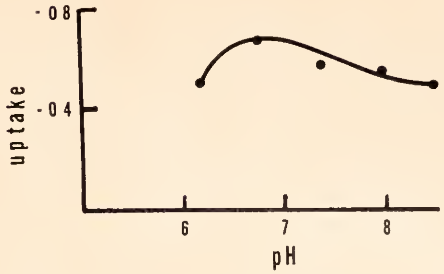

FIGURE 3. The uptake of ^{14}C-palmitate (0.05 mM) at various pH values. Each point is the mean of 8 replicates.

occurred at such inhibitor concentrations. This would explain the high K_i value for linolenate and the fact that it was not possible to carry out a Lineweaver-Burk plot with lignocerate. In other words, inhibition of palmitate uptake can occur in one of two ways, either by an inhibitor that interacts with the transport carrier site over its entire concentration range, or, by an inhibitor that, at high concentrations at least, alters the physical properties of the incubation medium and thus interferes with palmitate availability at the carrier site.

Effects of pH on palmitate uptake

Examination of palmitate uptake from incubation media buffered to a range of pH values with tris-maleic acid buffer (Fig. 3) indicated the occurrence of a pH optimum value, 6.8. The optimum pH appeared to be independent of palmitate concentration (Fig. 4).

Effects of bile salts on palmitate uptake

Sodium desoxycholate was included in incubation media at a concentration of 1 μMole/ml. Substitution of taurocholate for desoxycholate had no effect on palmitate uptake; this was examined over a wide range of exogenous palmitate concentrations.

Experiments were carried out to determine a possible role of bile salts in the transport of palmitate. In the complete absence of desoxycholate, palmitate uptake decreased 9.2% to 52.7%, depending upon palmitate concentration in the medium (Table II, column 1). The effects of increasing desoxycholate concentration on

TABLE II

Per cent inhibition of ^{14}C-Palmitate uptake in the absence of sodium desoxycholate

Concentration of Palmitate	Per cent inhibition using diluted 2 mM Palmitate stock	Per cent inhibition using diluted 10 mM Palmitate stock
0.025 mM	9.2	19.1
0.100 mM	26.4	23.1
0.200 mM	52.7	26.5
0.800 mM	30.0	18.4

palmitate uptake are shown in Figure 5. Varying the concentrations from 0.025 m*M* to 2.0 m*M* desoxycholate did not alter palmitate uptake, but concentrations of bile salt in excess of 2.0 m*M* brought about a twofold increase in the transport of palmitate. The effects of increasing the concentration of desoxycholate above 3.0 m*M* could not be examined due to the formation of an insoluble precipitate.

*The use of a 10 m*M *palmitate stock solution*

In order to extend the range of concentrations over which the uptake of ^{14}C-palmitate could be investigated, a 10 m*M* palmitate stock solution was made up (previous data refer to dilutions of a 2 m*M* palmitate stock). The uninhibited uptake of palmitate diluted from the stronger stock solution is shown in Figure 1, curve b. It is evident that, at all concentrations, the palmitate uptake from a diluted 10 m*M* stock is greater than that from a diluted 2 m*M* stock (curve a). The 10 m*M* curve also showed non-linearity and, hence, a K_t (1.0 m*M*) and Vmax (1.4 μMoles/g/2 min) could be determined.

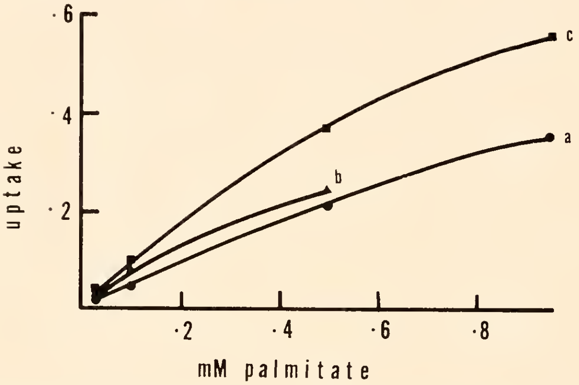

FIGURE 4. The uptake of ^{14}C-palmitate at pH 6.2 (Curve b), pH 6.8 (Curve c) and pH 8.5 (Curve a) as a function of palmitate concentration. Each point is the mean of 8 replicates.

Because of the differences demonstrated between uptake from dilutions of stocks of different strengths, the effects of various higher fatty acids were investigated using diluted 10 m*M* stock palmitate. The results of these experiments are shown in Table III. At concentrations of 1.0 m*M*, all fatty acids investigated, with the exception of heptadecanoate, were more effective inhibitors of palmitate uptake when dilutions from 10 m*M* stock solutions were used than was the case with dilutions from 2 m*M* stock solutions. Furthermore, oleate, at a concentration previously shown to be stimulatory (*i.e.*, at the same concentration as palmitate) failed to stimulate palmitate uptake when the 10 m*M* dilution was used. The effects of depletion of the incubation medium of desoxycholate on palmitate uptake were investigated using 10 m*M* dilutions. The results of this experiment are shown in Table II, column 2; these results are expressed as per cent reduction in palmitate uptake as a result of omitting the bile salt. At 0.025 m*M* palmitate the absence of bile induced a greater reduction of palmitate uptake with the 10 m*M* dilution than

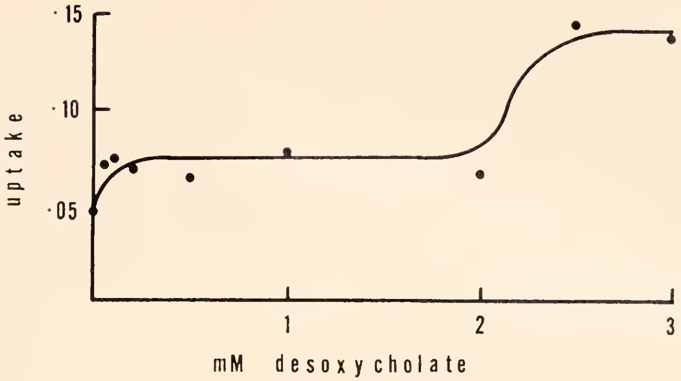

FIGURE 5. The effects on the uptake of ^{14}C-palmitate (0.05 m*M*) of increasing concentrations of sodium desoxycholate. Each point is the mean of 8 replicates.

with the 2 m*M* dilution. At all other palmitate concentrations, this effect was reversed. With both dilutions, the maximum reduction of uptake due to the absence of desoxycholate occurred at 0.2 m*M* palmitate.

Attempts were made to demonstrate differences in the physical properties of palmitate solutions diluted from 2 m*M* and 10 m*M* stock solutions. No osmotic pressure differences, using depression of freezing point method, could be shown. Ultra-centrifugation did not reveal any differences in physical properties and the use of fat-soluble dyes proved to be uninformative.

DISCUSSION

Arme and Read, (1968) have reviewed the literature concerning the entry of fatty acids into animal tissues. These workers have presented evidence for the mediation of transport of acetate and other short chain fatty acids through the tegument of the tapeworm *Hymenolepis diminuta*. The tegumentary site at which mediation occurred was shown to be specific for saturated fatty acid salts containing less than 9 carbon atoms in the hydrocarbon chain. The present investigation has provided evidence for the existence of a second fatty acid transport system in

TABLE III

*Effect of various fatty acids on the uptake of ^{14}C-Palmitate (0.025 m*M*) diluted from a 10 m*M* stock*

Addition	Ratio addition to Palmitate	Uptake ± Standard error	N	P	% Effect	Effect
None	—	0.044 ± 0.001	8	—	—	—
Decanoate	40:1	0.029 ± 0.001	8	<0.001	34.1	Inhibition†
Heptadecanoate	40:1	0.022 ± 0.001	8	<0.01	49.0	Inhibition†
Oleate	1:1	0.046 ± 0.003	4	>0.8	3.1	None
Linoleate	40:1	0.023 ± 0.002	4	<0.001	46.8	Inhibition†

† Considered to be significant by application of Student's t test.

H. diminuta. This system is concerned with the transport of higher fatty acids, and seems to be specific for saturated and unsaturated long chain fatty acids.

Although only ^{14}C-palmitate has been used as a substrate for the investigation of long chain fatty acid transport, it is not unreasonable to postulate that the various higher fatty acids, shown to be competitive inhibitors of palmitate transport, are themselves transported via the same carrier site. The affinities of these inhibitory acids for the palmitate site are reflected by their inhibitory efficacy and their inhibitor constants. It would appear that those acids most closely resembling palmitate, in terms of chain length, have the greatest affinities for the palmitate transport site. The high per cent inhibitions of palmitate uptake by lignocerate and linolenate are thought to be due, in part, to their physical effects on aqueous solutions of palmitate, particularly when the former acids were present at high concentrations, rather than simply attributable to their interaction with the carrier site. In this way the palmitate site is similar to the acetate-butyrate site in that the site affinity shown by a fatty acid inhibitor is related to the difference between the number of carbon atoms in the substrate molecule and the number of carbon atoms in the inhibitor molecule.

The transport of palmitate by *H. diminuta* resembles the transport of short chain fatty acids (Arme and Read 1968) and of purines and pyrimidines (MacInnis, Fisher and Read, 1965) in that two component pathways were detectable. At low concentrations of palmitate, up to 4.5 m*M*, diffusion through the tegument appears to be lesser than mediated transport, as shown by saturation kinetics. On the other hand, experiments carried out using inhibitors at concentrations far in excess of palmitate concentration have shown that inhibition never interrupted more than 70% of palmitate uptake. Furthermore, the remaining 30% uptake (in fact, the remaining 42.5% palmitate uptake subsequent to inhibition with heptadecanoate) was found to be linear with respect to palmitate concentration, and therefore thought to be diffusion. It would seem that contradictory data exist; the saturation data indicate that at 0.25 m*M* little palmitate enters by diffusion, whereas inhibition data indicate that, at the same concentration, over 40% of palmitate uptake is via a diffusion system. This discrepancy might be resolved if it is postulated that the inhibitor, in this case heptadecanoate, interacted with palmitate at the carrier site, thus precluding mediated transport, but had no effect on the diffusion of palmitate, the latter being masked by mediated transport in the absence of inhibitor.

It is of interest to compare the rate of entry of acetate and palmitate through the tegument of *H. diminuta.* Arme and Read (1968) showed the uninhibited uptake of acetate to be 0.105 μMoles/g/min from a 0.1 m*M* solution; a rate of 0.210 μMoles/g/2 min is derived by multiplication. Under identical conditions, the uninhibited uptake of palmitate was measured as 0.074 μMoles/g/2 min. Jacobsen and Fairbairn (1967) demonstrated that the label from ^{14}C-acetate was incorporated into *H. diminuta* lipids at a lower rate than that of higher fatty acids, including ^{14}C-palmitate. Arme and Read suggested that the relatively low incorporation rate of acetate might be due to dilution with endogenously-produced acetate. This would seem to be highly probable in the light of the present demonstration that during two minutes, palmitate is transported at a rate about one-third that of acetate. It is worthy of note to record that Winterbourn and Batt (1968) observed a "high incorporation" rate of plasma ^{14}C-palmitate into the lipids of bovine leukocytes. It would be of interest to know the relationship between the rates of incorporation and uptake in the latter system and also to compare these with rates of incorporation and uptake of acetate.

A number of workers investigating membrane transport have observed that apparent synergism may occur when two chemically similar substances are included in a single incubation medium. Schafer and Jacquez (1967) observed that the uptake of L-tryptophan was stimulated in the presence of equimolar concentrations of either L-leucine, DL-p-fluorophenylalanine or L-methionine. These authors considered these synergistic phenomena to be "competitive stimulations." Previously, Jacquez (1961, 1963) recorded that while a number of neutral L-amino acids would stimulate the uptake of L-tryptophan by Ehrlich ascites tumour cells, when the concentrations of both substrate and stimulator were equal, an increase in the concentration of the stimulatory amino acid induced inhibition of tryptophan uptake. Certain observations made in the present study are remarkably similar to those of Jacquez (1961, 1963). Oleate, linoleate, and linolenate, when present at the same concentration as palmitate, stimulated the uptake of palmitate by 31.4%, 25.2%, and 27.9%, respectively. When the concentrations of any one of these unsaturated acids was increased, inhibition of palmitate uptake occurred. Only one unsaturated fatty acid tested, palmitoleic, failed to stimulate palmitate entry at a 1:1 ratio.

It is considered that the above stimulations of palmitate uptake cannot be termed competitive stimulations since it has been shown, by application of the methods of both Dixon, and Lineweaver and Burk, that these three unsaturated fatty acids are, in fact, competitive inhibitors of palmitate transport. On the other hand, it was demonstrated that laurate would stimulate palmitate transport irrespective of its concentration in the medium and that, furthermore, stimulation was related to laurate concentration. Again, by application of Dixon's method, laurate was shown to be a partially competitive stimulator of palmitate uptake. MacInnis *et al.* (1965) observed that thymine competitively stimulated the transport of uracil by *H. diminuta.* They considered that this phenomenon might be accounted for either by the prevention of uracil efflux by thymine or by the combined effect of one molecule of uracil and one molecule of thymine at the carrier site. They further pointed out that their data did not exclude possibility of dimerisation as a prerequisite of membrane transport.

The two types of stimulation discerned in the uptake of palmitate do not seem to be related. Laurate is known to increase the solubility of fatty acids in organic solvents (Fieser and Fieser, 1959), a property apparently unique to this fatty acid. Its stimulatory effect on palmitate transport may, therefore, reflect its effect on the solubility of palmitate rather than a direct effect on the carrier site. No explanation can, at present, be offered to account for the stimulation of palmitate uptake by three unsaturated fatty acids, especially since palmitoleate failed to be stimulatory. All four unsaturated fatty acids investigated occur in the Cis form (Heilbron, Cook, Bunbury and Hey, 1965) thus ruling out certain stereo-chemical effects.

The effects of bile salts on palmitate transport have been examined in the present study. Sodium desoxycholate is known to form specific intermolecular complexes with fatty acids; such compounds are termed choleic acids (Fieser and Fieser 1959). The formation of a bile-fatty acid complex (not to be confused with a micelle) markedly increases the solubility of the fatty acid and it was thought that this was possibly the way in which desoxycholate could increase the uptake of palmitate. However, evidence has been accumulated which suggests that, under the experimental conditions described, choleic acids are not formed, or if they are, they exert no effect on palmitate transport. Apparently taurocholate does not form intermolecular inclusion complexes (Fieser and Fieser 1959), and it was found that taurocholate could replace desoxycholate without altering palmitate uptake. Choleic acids are composed of a specific number of molecules of desoxycholate enclosing a single molecule of fatty acid. In the case of palmitate, eight molecules of desoxycholate are required for each palmitate molecule. Therefore, if choleic acid formation is a prerequisite of optimum uptake, the initial ratio of desoxycholate to palmitate in the medium will be critical. However, this has not been found to be so (Fig. 5). The twofold increase in palmitate entry recorded when the concentration of desoxycholate exceeded 2.0 m*M* may be accounted for in one of two ways. First, the tegument of the worm may be rendered more permeable to palmitate due to the direct effect of relatively high levels of bile salt, or second, at such concentrations, the bile salt may be existing in predominantly micellar form (Bailey and Fairbairn 1968). Above its Critical Micelle Concentration, a surfactant, such as bile salt, will have distinct effects on aqueous solutions of fatty acids, particularly in the direction of solubilization. In contrast to palmitate, the uptake of ^{14}C-acetate was unaffected by desoxycholate, irrespective of the concentration of the latter. This may be taken as evidence that bile salt exerts its influence at higher concentrations in micellar form.

In the mammalian jejunum, bile salt (taurodeoxycholate) is absorbed at a rate similar to oleate (Gordon and Kern, 1968) indicating the existence, and importance to fatty acid transport, of bile salt-lipid micelles. Nonetheless, with the exception of the experiments employing unusually high levels of bile salt, there is no evidence that micelles exist in the present incubation media. Application of the expression:

$$\log \text{Critical Micelle Concentration} = A - B.nC,$$

[where A and B are physical constants for homologous series of fatty acid salts and nC is the number of carbon atoms in the hydrocarbon chain (Osipow, 1962)], has indicated that, under the experimental conditions used, the CMC for palmitate is 18.7 m*M*.

The observation that palmitate uptake from a solution of given strength varies according to the strength of the stock solution from which it was diluted, has proved difficult to interpret. It is unlikely that a 0.025 m*M* palmitate solution made by diluting a 2 m*M* stock is qualitatively different from a 0.025 m*M* solution made by diluting a 10 m*M* stock; it is reasonable to suppose that these two solutions differ only in the proportions of a particular moiety. It was originally conjectured that the 10 m*M* stock solution of palmitate contained a greater number of micelles than did its weaker counterpart, and that micellar fatty acid was transported more efficiently than the ionic form. However, it has become evident that neither of the stock palmitate solutions contained micelles, at least in significant number. At concentrations below their critical micelle concentration aqueous solutions of amphipaths contain free ions in equilibrium with small aggregates (Osipow, 1962). Little is apparently known about the size or preponderance of these aggregates at various amphipath concentrations. It is, therefore, postulated that the 10 m*M* stock palmitate solution contains either a greater number of molecular aggregates or that these aggregates are of larger size than are found in the 2 m*M* solution. Dilution of these stock solutions apparently does not alter the nature of the aggregates. Thus, while both 2 m*M* dilutions and 10 m*M* dilutions, at the same final concentration of palmitate, are ostensibly identical, they possess physical dissimilarities that are manifested in differing rates of palmitate transport. In no way has it been possible to demonstrate the actual nature of these differences between the two palmitate solutions. In all cases but one, the effects of other fatty acids on the transport of palmitate diluted from a 10 m*M* stock have been to increase inhibition of uptake or lower stimulation, depending upon the particular additional fatty acid. All solutions of fatty acids, other than labeled palmitate, were made up to contain 5 μMoles/ml. It is probable that these solutions also contain ions and aggregates, and that they will differ, in terms of number or size of these aggregates, from both the 2 m*M* and 10 m*M* diluted solutions when they, themselves, are diluted. Hence, if aggregated molecules of palmitate are more rapidly transported, it might be expected that aggregated molecules of other fatty acids might act more efficiently as inhibitors than solutions containing less or smaller aggregates.

A further piece of evidence for the relationship between fatty acid transport and the presence of molecular aggregates lies in the quantitatively different effects on palmitate uptake when diluted 2 m*M* and 10 m*M* stocks are used in the absence of bile salt. At all but the lowest palmitate concentrations (Table II) the effect of removing bile salt from the incubation media was less pronounced when the medium contained a 10 m*M* dilution. If it is postulated that bile salt, even at concentrations below its critical micelle concentration, functions as an aggregator of fatty acid, then the presence of preformed aggregates of palmitate might be expected to diminish the effects of depleting and removing bile salt from the incubation media.

It is thought that aggregates of palmitate are unlikely to be transported in their entirety, but serve merely to bring a greater number of fatty acid molecules within the proximity of the membrane carrier sites than would occur if ions existed independently. That is to say that, when a single molecule, as part of a polymolecular aggregate, attaches to the carrier site, it predisposes other molecules of the aggregate for transport.

The effects of pH on palmitate uptake are reminiscent of the pH effect on an enzymic reaction, in that a pH optimum was detected. It has been calculated that sodium palmitate is more than 90% ionized in the pH range of this investigation and consequently the observed pH optimum is somewhat difficult to interpret.

The present investigation has shown that the tapeworm *H. diminuta* possesses a fifth type of mediated transport system situated on or in the tegument. The higher fatty acid transport site resembles other transport systems in its degree of specificity, whereas the differences that have been demonstrated are thought to represent the physical properties of aqueous solutions of fatty acids and bile salts rather than intrinsic differences in the carrier site.

SUMMARY

1. The uptake of ^{14}C-palmitate by *Hymenolepis diminuta* has been shown to occur via a mediated process at concentrations of up to 4.5 m*M* palmitate. Entry

of palmitate by diffusion is not thought to be significant under present conditions.

2. Fatty acids containing less than 12 carbon atoms and substances other than fatty acids did not inhibit palmitate uptake. Laurate, at all concentrations, stimulated uptake, while saturated fatty acids with up to 24 carbon atoms inhibited uptake to varying degrees. Three of the four unsaturated fatty acids tested stimulated palmitate uptake when present at concentrations equal to that of palmitate and inhibited uptake when their concentrations were increased. All saturated fatty acids that affected palmitate uptake were found to be partially competitive inhibitors or stimulators.

3. A pH optimum was detected for palmitate uptake.

4. The effects of bile salts on palmitate uptake were investigated, but the role of bile in the transport of palmitate could not be elucidated.

5. The differences in palmitate uptake following dilution of 2 m*M* and 10 m*M* palmitate stock solutions are thought to reflect the physical properties of fatty acid solutions. It is postulated that 10 m*M* dilutions of palmitate contained either a greater number of, or larger, aggregated molecules than were present in 2 m*M* dilutions. It is considered that enhancement of palmitate uptake is related to the presence of these molecular aggregates.

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THE ENVIRONMENTAL AND HORMONAL CONTROL OF GROWTH AND REPRODUCTION IN THE ADULT FEMALE STONE CRAB, *MENIPPE MERCENARIA* (SAY)[1]

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The growth and reproductive biology of the adult female stone crab, *Menippe mercenaria*, an economically important species in Florida, have never been investigated in the laboratory. These morphogenetic processes are influenced both by hormones and by seasonal changes in the environment. This study seeks to survey the relation between these processes, their endocrine control, and the changing environmental conditions provided in nature. Special attention has been paid to the interrelationship between growth and reproduction. This aspect has been studied by Bauchau (1961), Cheung (1966) and Démeusy (1964, 1965a, 1965b and 1965c) in *Carcinus maenas*, a boreal species. The present study employs adult female specimens of *M. mercenaria* since they continue to grow after reaching sexual maturity, unlike *Callinectes* in which growth ceases at maturity (Truitt, 1939).

The relationship between growth and reproduction was defined by (1) observing the occurrence of molting and spawning in a sample of the wild population; and (2) removing the eyestalks at different stages of the molting cycles and during intermolt periods occurring at different times of the year, because the eyestalks contained endocrine factors regulating these processes. Results of this study also yielded information on the life history of this species.

MATERIALS AND METHODS

Adult stone crabs were collected in baited traps (opening 3 × 5 inches) which were set in Biscayne Bay, approximately one mile from the Institute of Marine Science, University of Miami. Only crabs above 45 mm in carapace width (CW) were used since mature ones had not been found below this size. In the laboratory, they were maintained in circulating sea water in glass tanks with slate bottom, each measuring 1 × 2 feet in area, or in wooden tanks, each with about the same volume as the glass aquaria. The crabs were isolated from each other in order to avoid loss through cannibalism. Crabs held in glass aquaria received normal room daylight supplemented by fluorescent lamps; those in the wooden tanks were exposed to normal room daylight without supplementation. The circulating water in the laboratory was pumped almost directly from the Bay, so that its fluctuations in salinity and temperature were found to follow closely the same patterns as those in nature. Experimenting under controlled systems of constant salinity or temperature had been considered, but was rejected here in preference to the circulating sea water, since the former method could not yield useful information for the study of life history.

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All the crabs were fed daily with approximately 4 grams of shrimp meat; the tanks and compartments were cleaned regularly. Each tank was examined daily to record molting, spawning, or death in the normal, as well as experimental, animals.

Eyestalks were removed after the method of Cheung (1964) adopted from Bliss (1953). This included anesthetizing the crabs by cooling, and carefully cauterizing. This technique, when performed rapidly, almost eliminated post-operative mortality.

The data describing the natural environment were based on water samples collected under the pier of the Institute. Similar data on salinities and temperatures were recorded daily from the laboratory tanks. Figure 1 gives the salinities and temperatures of the circulating water during the study period, which were found to be close to the figures obtained from water samples under the pier.

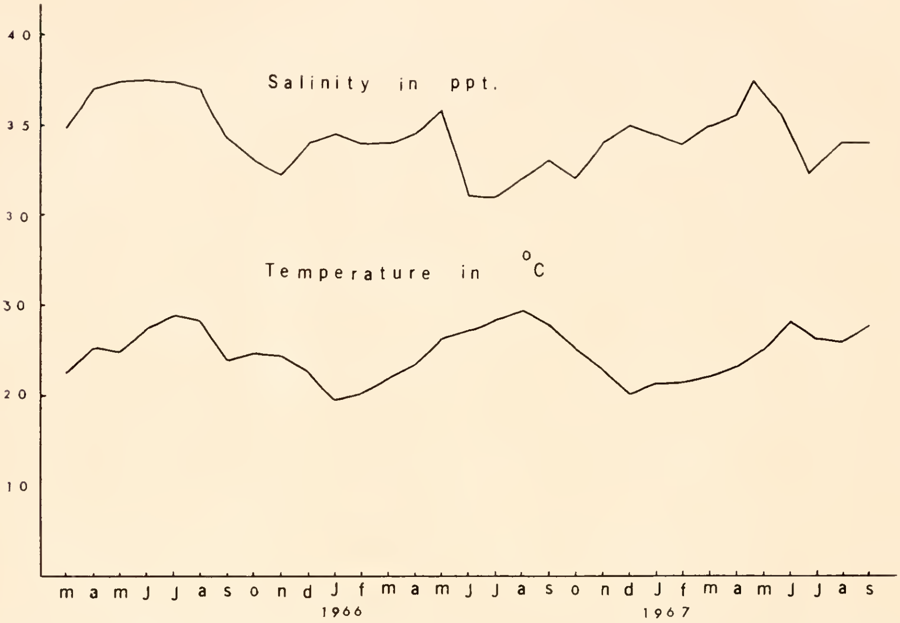

Figure 1. Salinity and temperature curves over period of study. These were monthly averages.

Results

Environmental factors

Since the same food was given daily to every crab, difference due to this factor was ruled out. No significant correlation could be found in spawning or molting with the lunar phase. The data may not be adequate to demonstrate this, or possibly, during prolonged exposure to laboratory conditions, the animals may have lost their rhythm. Therefore, this work has neither proved nor disproved the existence of a lunar periodicity.

The average monthly temperature ranged from 19.5° to 29° C during the study, with similar seasonal patterns in both years. As the spawning season had a more definite pattern, it is natural to assume that this would be related to temperature changes. Further, as changes in light intensity and photoperiod are related to those of temperature, the effects of light and temperature were distinguished whenever possible.

The average local salinity over the two-year span of this study varied between 29 and 38‰. Further, the seasonal changes in salinity during the two years were of an entirely different pattern, so that this factor has no apparent correlation with seasonal variations in spawning frequencies, which exhibited a similar pattern during the two years. The effects of salinity on molting, which displayed no regular seasonal pattern, are suggested in this crab (Noe, 1967) as well as certain other crabs, when the works of Bliss and Boyer (1964) and De Leersnyder (1967) are considered.

The best studies on environmental effects in this work are thus limited to temperature and light.

TABLE I

Data on molting and spawning of one female crab, which spawned the greatest number of times (13) yet known between two ecdyses

Molting: December 1, 1965; December 4, 1966.
Spawning: February 12, 1966; March 14, 1966; April 8, 1966; May 2, 1966; May 20, 1966; June 7, 1966; June 27, 1966; July 13, 1966; July 31, 1966; August 16, 1966; Sept, 2, 1966; Sept. 20, 1966; Oct. 4, 1966.

Endocrine control

The eyestalks are of particular interest not only because they contain the X-organs generally recognized to be responsible for the production of both molt-inhibiting (MIH) and ovarian-inhibiting (OIH) hormones (Carlisle and Knowles, 1959; Passano, 1960a) but because it is through them that visual stimuli are received. Thus they act as an "interface" between many external factors that may affect the MIH as well as OIH. The destalking experiments that follow are an attempt to study some relationships between the factors of external and internal environments.

Molting stages

While destalking of intermolt decapods has been found to accelerate molting, no acceleration due to the operation occurs if the animal is destalked during the premolt stage (Drach, 1944). There is little doubt that this generalization applies to all decapods. No results of destalking postmolt crabs have been published, probably because they are not easy to collect in sufficient numbers for experiments. During this study, however, a small number of crabs molted in the laboratory. These were destalked, with results to be described below.

Ecological results

Seasonal occurrence Between April, 1965 and February, 1967, 27 adult female crabs were maintained in the laboratory. Spawning and molting records in this group of animals were kept but not published here. They show that spawning was much more frequent than molting. Within a single intermolt period several spawnings could occur (Table I). Records of spawning and molting for the same animals during the experimental period are given in Figure 2, showing that they spawned 126 times, but molted only 29 times. There was thus an average of 4.5 spawnings within one single molt cycle in the laboratory. Further, the histogram representing spawning has its peak in the months of August and September. In October, spawning declined sharply. Spawning was at its lowest

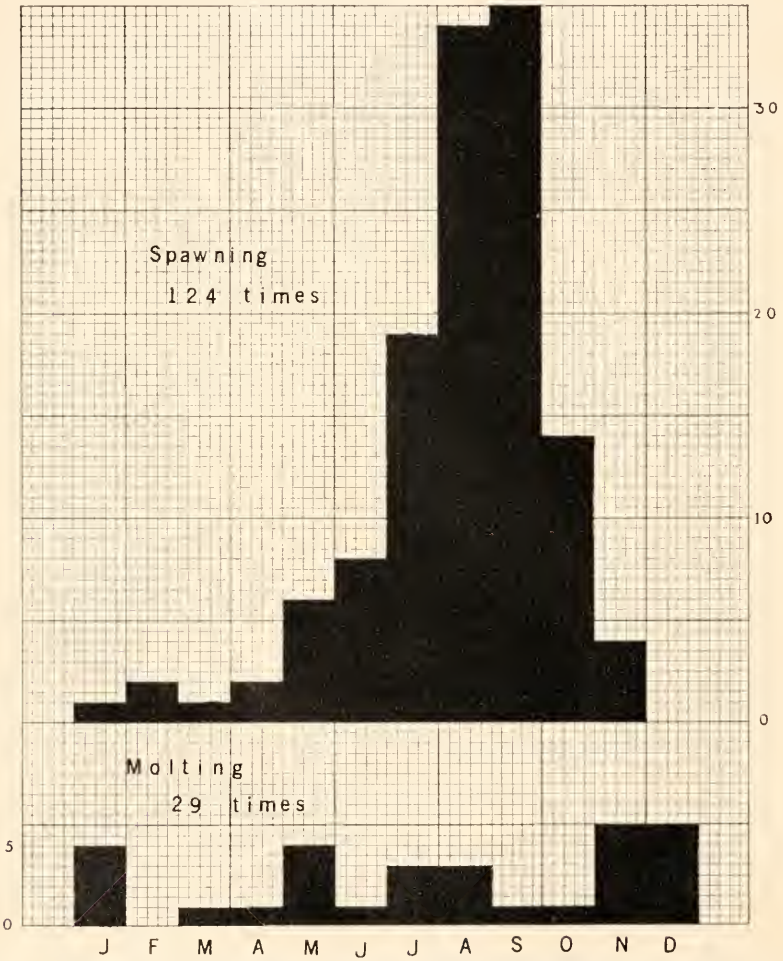

FIGURE 2. Histograms summing up records of spawning and molting for a group of stone crabs reared in the laboratory over a year under conditions described in the text.

TABLE II

All the female crabs listed in this Table spawned more than twice during the period of study. The position of "x" for each crab indicates the month in which the shortest inter-spawning period of the particular crab occurred. The total number of shortest inter-spawning periods in each month was derived by adding up the number of "x"s occurring in the month. Whenever the "x" lay between two months, one-half of it is credited to each month.

Crab no.	Jan.	Feb.	Mar.	Apr.	May	Jun.		Jul.		Aug.		Sep.		Oct.	Nov.	Dec.
1						x		x		x						
2							x									
4											x					
5b							x				x					
6													x			
7b													x			
8a											x					
10											x	x				
11											x					
12										x						
13								x				x				
14a												x				
15									x							
16									x							
18												x				
20										x						
21										x						
23									x							
28										x						
29											x					
30					x											
Total					1	2		4.5		9.5		8		1		

ebb in winter, in the months of November through March. This pattern is in good agreement with the results of sampling (Noe, 1967) in the same locality where these experimental animals were caught, during a period that overlapped the present experiments.

Of the 29 ecdyses of the above animals, 14 occurred between November 1 and January 31, in the same period when spawning least occurred. The other 15, however, were scattered irregularly over 7 months in the rest of the year. The molting season is therefore not as clearly defined as that of spawning. Because of the fact that temperature, salinity and photoperiodic conditions in the laboratory were similar to those in nature, it is reasonable to expect that the molting and spawning seasons of the laboratory population should agree with the field data.

Intervals between molting and spawning Ovarian development and molting are antagonistic processes in this species (Noe, 1967) as they are in *Carcinus maenas* (Bauchau, 1961; Cheung, 1966; Démeusy, 1963). Thus the length of the period between molting and spawning may indicate the rate of change in the physiological conditions of the animals; the particular seasons at which they occur may also reflect influence of seasonal factors.

Sixteen crabs were available for this study. The results are shown in Figure 3. All the spawnings took place at the C4 stage of the integument.

It is noteworthy that all the spawnings, with one exception, occurred in the

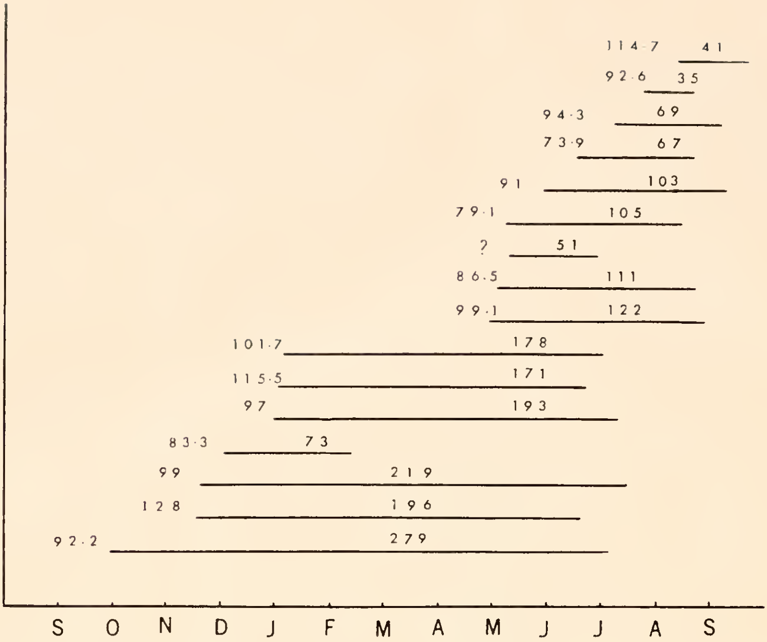

FIGURE 3. Observed periods from molting to spawning in 16 crabs reared in the laboratory. The number on the left side of each line represents the carapace width of a crab in millimeters. The number above each line indicates the number of days from molting to spawning.

summer months irrespective of the month in which the previous molt had taken place. This suggests that ovarian development depends on seasons and is independent of the length of time since the last molting occurred. The interval between molting and spawning bears no correlation with the size of the animals.

Since several spawnings could take place within one molt cycle, the comparative lengths of inter-spawning periods in any single animal reveal different rates of ovarian development at different times of the year.

Twenty-one crabs spawned more than twice within the same molt cycle. The distribution of the shortest inter-spawning intervals in time is illustrated in Table II. When the interval includes parts of two successive months, half of the period is considered to be in each of the two months. A single shortest inter-spawning period occurred in May, 2 in June, 4.5 in July, 9.5 in August, 8 in September and 1 in October. In the rest of the year there was no shortest inter-spawning period.

These results suggest that ovarian growth is most accelerated during the warmest month (average temperature 29° C) but not necessarily the month with longest day-length or highest light intensity. Any effect of light is probably restricted to the initial phase of ovarian development.

Data on 15 crabs were available for this study (Figs. 4). With one exception, the last spawnings were confined to autumn. The subsequent moltings, however, had a wide scatter over all seasons, ranging from late October to middle August of the next year, although the majority took place in winter. The intervals between the last spawnings and the subsequent moltings bear no correlation with the size of the crabs.

Only three adult female crabs molted twice during the period of study. One required 6 months, while the other two, 1 year. This information suggests that intermolt periods for the adult stone crabs are long.

Relationship between spawning and molting In Figure 2, the difference between the distribution of spawning and ecdysis is noteworthy. The histogram of spawning shows a normal distribution with a peak in August or September, suggesting that summer is the most favorable season. Moltings, on the other hand, show no definite peak, although about half of them occurred in the winter months. If winter were favorable for growth, as summer appears to be for spawning, the data should appear as a normal distribution curve with a peak in winter. But this relationship did not occur even though molting and ovarian development are antagonistic processes. There was no inverse correlation between the histograms. Further, from Figures 3 and 4, molting did not appear to be as dependent on season as spawning. Possibly it was affected by salinity changes which appeared rather irregularly in this area.

Conclusions from ecological observations Ovarian development of the crab was closely correlated with local water temperature. Optimal development was

TABLE III

Destalking experiment on adult female stone crabs kept in wooden tanks, August, 1966

Crab no.	CW (mm)	Date destalked	Date molted	Date spawned
4	54	Aug. 31, 1966	Sep. 19, 1966	Oct. 11, 1966
5	53	Aug. 31, 1966	Sep. 25, 1966	Oct. 18, 1966
6	71	Aug. 31, 1966	Oct. 12, 1966	—
7	92	Aug. 31, 1966	Oct. 1, 1966	—
8	72	Aug. 31, 1966	Sep. 23, 1966	Oct. 18, 1966 Jan. 3, 1967 Feb. 28, 1967
9	73	Aug. 30, 1966	Oct. 8, 1966	—
10	70	Sep. 1, 1966	Sep. 29, 1966	—
12	90	Aug. 31, 1966	Oct. 11, 1966	—
13	84	Aug. 31, 1966	Oct. 11, 1966	—
11	105	Aug. 31, 1966	Oct. 11, 1966	—
F9	87	—	—	—
F10	80	—	—	—
F11	72	—	—	—
F12	74	—	—	—
F13	76	—	Oct. 27, 1966	—
F14	95	—	—	—
F15	78	—	—	—
F16	45	—	—	—
F17	94	—	—	—
F18	97	—	—	—
F19	75	—	—	—
F20	64	—	—	—
F21	102	—	—	—

Table IV

Destalking experiment on adult female stone crabs kept in wooden tanks, November, 1966

Crab no.	CW (mm)	Date destalked	Date molted	Date spawned
F1	67	Nov. 2, 1966	Dec. 8, 1966	—
F2	94	Nov. 2, 1966	—	—
F3	95	Nov. 2, 1966	Jan. 1, 1967	—
F4	71	Nov. 2, 1966	Nov. 28, 1966	Jan. 18, 1967
F5	88	Nov. 2, 1966	Dec. 27, 1966	—
F6	55	Nov. 2, 1966	Dec. 10, 1966	—
F7	85	Nov. 2, 1966	Nov. 28, 1966	—
F8	77	Nov. 2, 1966	Dec. 27, 1966	—
F9	87	Nov. 2, 1966	Dec. 16, 1966	Feb. 20, 1967 Apr. 11, 1967 May 20, 1967
F10	80	Nov. 2, 1966	Dec. 19, 1966	Feb. 18, 1967 Apr. 9, 1967
F11	72	Nov. 3, 1966	Dec. 25, 1966	Mar. 28, 1967
F12	74	Nov. 3, 1966	Dec. 5, 1966	—
F14	95	Nov. 3, 1966	Dec. 12, 1966	Feb. 24, 1967
F15	78	—	—	—
F16	45	—	—	—
F17	94	—	—	—
F18	97	—	—	—
F19	75	—	—	—
F20	64	—	—	—
F21	102	—	—	—
F22	87	—	—	—
F23	82	—	—	—
F24	88	—	—	—
F25	114	—	—	—
F26	59	—	—	—
F27	94	—	—	—
F28	70	—	—	—
F29	67	—	—	—

seen at approximately 28° C. Longest day-length and highest light intensity do not correspond with fastest maturation of eggs. The age (as reflected by size) of the crab has no relation to the length of the developmental period of the eggs. Further, the spawning season is not affected by the time when the previous molting occurred. At the lowest ebb of spawning in winter, molting occurred in abundance (an exception to this was in February, the coldest month which is apparently unfavorable for either molting or spawning). These results suggest that growth was inhibited by reproductive activities but not *vice versa*. To clarify factors controlling growth and reproduction the following experiments were performed.

Seasonal changes in the morphogenetic effects of the eye-stalks at intermolt stage

MIH as well as OIH are produced by the eyestalk X-organs. Removal of the eyestalks has been found to cause either precocious molting or ovarian development in various species (Passano, 1953; Brown and Jones, 1949; Démeusy, 1962).

TABLE V

Destalking experiment on adult female stone crabs kept in wooden tanks, March, 1967

Crab no.	CW	Date destalked	Date molted	Date spawned	Dead
F17	94	Mar. 24, 1967	May 14, 1967	—	
F19	75	Mar. 24, 1967	Apr. 26, 1967	—	
F21	102	Mar. 24, 1967	—	—	Apr. 5, 1967
F23	82	Mar. 24, 1967	May 6, 1967	—	
F24	88	Mar. 24, 1967	—	Apr. 25, 1967	
F26	59	Mar. 24, 1967	Apr. 21, 1967	—	
F29	67	Mar. 24, 1967	Apr. 30, 1967	—	
F15	78	—	Jun. 7, 1967	—	
F16	45	—	Apr. 12, 1967	—	
F18	97	—	—	—	
F20	64	—	Jun. 10, 1967	—	
F22	87	—	May 25, 1967	—	
F25	114	—	—	May 4, 1967	
F27	94	—	—	—	
F28	70	—	May 22, 1967	—	

TABLE VI

Destalking experiment on adult female stone crabs kept in glass tanks, February, 1967

Crab no.	CW	Date destalked	Date molted	Date spawned
5	128	Feb. 7, 1967	Mar. 5, 1967	—
6	114.7	Feb. 7, 1967	—	Mar. 14, 1967 Apr. 13, 1967 May 16, 1967
10	86.5	Feb. 7, 1967	Mar. 30, 1967	Apr. 30, 1967 Jun. 5, 1967
11	101.7	Feb. 7, 1966	—	Mar. 14, 1967
14	94.3	Feb. 7, 1967	—	Mar. 11, 1967
15	97	Feb. 7, 1967	Apr. 1, 1967	—
23	115.5	Feb. 7, 1967	—	Mar. 14, 1967
C	74	Feb. 7, 1967	Mar. 20, 1967	Apr. 27, 1967 Jun. 20, 1967
B		Feb. 7, 1967	Mar. 30, 1967	—
4	91	—	—	Apr. 30, 1967 May 20, 1967 Jun. 5, 1967
G	81	—	—	May 12, 1967 May 26, 1967 Jun. 5, 1967
7	99.1	—	—	Apr. 28, 1967
29	92.6	—	—	Apr. 21, 1967
20	79.1	—	—	Jun. 18, 1967
H	73	—	—	—
13	92.2	—	—	May 21, 1967
1	98.1	—	—	—
21	99	—	—	May 18, 1967 Jun. 1, 1967
34	91	—	May 12, 1967	—

These different responses have not been explained. Weitzman (1964) discovered that destalking specimens of *Gecarcinus lateralis* accelerated ovarian development in spring when such development normally occurs, but accelerated molting in the fall, when growth became dominant. This work indicated possible environmental influence on molting and maturation.

The following experiments on destalking stone crabs were designed to determine whether the activity of morphogenetic hormones varied with the change in seasons.

August to October, 1966 (Table III) All the crabs employed in this experiment were berried, and at the C4 stage (Passano, 1960a). The results show that destalking intermolt adult female crabs at this time invariably accelerated the subsequent molt.

November, 1966 to January, 1967 (Table IV) Both the destalked and control groups included control crabs from the last experiment as well as newly collected crabs. In the beginning of this experiment no crab was berried. Destalking at this time also caused an acceleration of molting. The reaction of both the old and the newly collected crabs was the same.

TABLE VII

Destalking experiment on adult female stone crabs kept in glass tanks, April, 1967

Crab no.	CW	Date destalked	Date molted	Date spawned
4	91	Apr. 18, 1967	—	Apr. 20, 1967 May 20, 1967 Jun. 5, 1967
G	81	Apr. 18, 1967	—	May 2, 1967
1	98.1	Apr. 18, 1967	—	—
21	99	Apr. 18, 1967	—	May 18, 1967 Jun. 1, 1967
34	91	Apr. 18, 1967	May 12, 1967	—
7	99.1	—	—	Apr. 28, 1967
29	92.6	—	—	Apr. 21, 1967 May 16, 1967
20	79.1	—	—	—
H	73	—	—	—
13	92.2	—	—	May 21, 1967

February to June, 1967 During this period, 3 experiments were performed. The first one (Table V) employed all the control crabs from the previous experiment. Fifteen crabs were available, of which 8 were held as controls, while the other 7 were destalked. Of the 7 destalked crabs, 5 responded by molting; one by spawning; and one died without yielding any results. Five of the control crabs molted, 1 spawned while 2 neither molted nor spawned during the period of observation.

During the second experiment (Table VI) the light intensity was increased while other environmental conditions were not different from those of the first experiment. The control group contained 10 crabs, the destalked group 9. During the period of observation, 5 destalked crabs molted, 4 spawned. Of these numbers 6 and 10 spawned more than once. One of the control crabs molted, 8 spawned, while 2 neither molted nor spawned. Again, some of them spawned more than once.

These 2 experiments yielded results different from those of the previous ones in that the crabs responded to destalking by spawning as well as molting.

The third experiment (Table VII) was also conducted with elevated levels of illumination identical with that in Table VI. There were 5 control and 5 destalked crabs. During the observation period, 3 of the destalked animals spawned (2 more than once), 1 molted and 1 yielded no results. Of the controls, 3 spawned, a reminder that this was the spawning season.

TABLE VIII

Destalking experiment on adult female stone crabs, September, 1967.
(Crabs kept in wooden tanks, above line; Crabs kept in glass tanks, below line)

Crab no.	CW	Date destalked	Date molted	Date spawned
B1	92	Sep. 20, 1967	—	Sep. 29, 1967
B2	96	Sep. 20, 1967	—	Oct. 4, 1967
B3	117	Sep. 20, 1967	—	Oct. 11, 1967 Oct. 24, 1967
B4	123	Sep. 20, 1967	—	Oct. 11, 1967
B5	96	Sep. 22, 1967	—	Oct. 4, 1967 Oct. 29, 1967
F15	88	Sep. 21, 1967	—	Oct. 13, 1967
F27	94	Sep. 21, 1967	—	Oct. 10, 1967
F28	82	Sep. 21, 1967	—	Sep. 29, 1967 Oct. 19, 1967
F24	98	Sep. 21, 1967	—	—
47	94	Sep. 5, 1967	—	Sep. 25, 1967
13	92.2	Sep. 5, 1967	—	Sep. 14, 1967 Sep. 26, 1967 Oct. 26, 1967
20	91	Sep. 5, 1967	—	Oct. 11, 1967
45	76	Sep. 5, 1967	—	Sep. 17, 1967
44	94	Sep. 5, 1967	—	Sep. 24, 1967 Oct. 16, 1967 Oct. 19, 1967
7	99.1	Sep. 5, 1967	—	Sep. 18, 1967 Oct. 6, 1967
29	92.6	Sep. 5, 1967	—	Sep. 15, 1967 Oct. 14, 1967
H	73	Sep. 5, 1967	—	Sep. 15, 1967
46	104	Sep. 5, 1967	—	Sep. 15, 1967

September 20th to November 5th, 1967 Eighteen crabs in two groups with different levels of illumination were destalked and observed. All operated crabs spawned (Table VIII), irrespective of the levels of illumination.

Conclusions to destalking experiments on intermolt crabs Two important points deserve attention: These results resemble those of Weitzman (1964) on *Gecarcinus* in that removal of the eyestalks may cause either spawning or molting in the adult female, depending on whatever process was dominant in the seasons when destalking was performed. (In this work, I am using spawning instead of ovarian growth as a criterion of reproduction.) In *Menippe,* between the time when molting was the dominant developmental process and when spawning replaced it, there was a transitional period (between January and September 1967) during which destalking might produce either molting or spawning, with the eventual complete dominance of spawning. The transition back to complete dominance of molting had not been followed, but would probably be more abrupt, judging by the fact that the limit of the spawning period is more sharply governed by the seasonal change (Figs. 3 and 4).

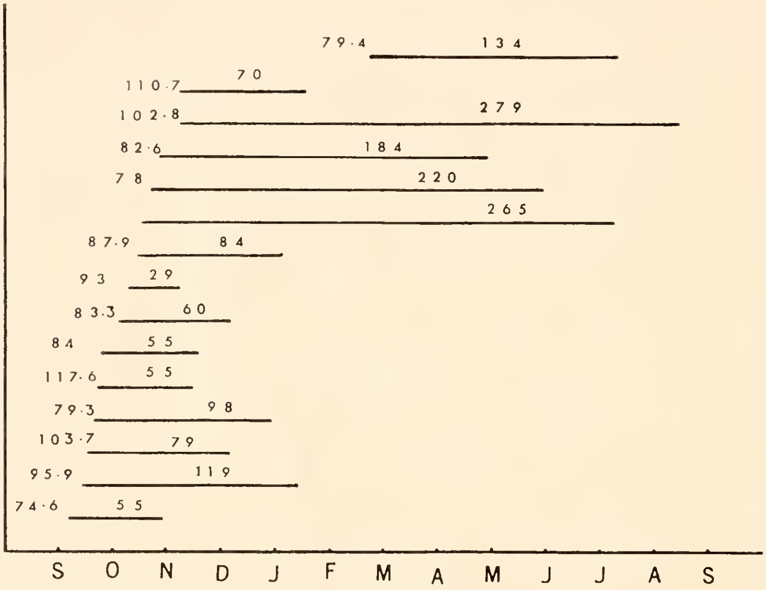

FIGURE 4. Observed periods from the last spawning to molting in 15 crabs reared in the laboratory. The number on the left side of each line represents the carapace width of a crab in millimeters. The number above each line indicates the number of days from spawning to molting.

Secondly, the response to destalking in the autumns of 1966 and 1967 was entirely different. During the first autumn, the response was molting whereas during the second, it was spawning. This together with the inconsistancy of the molting season possibly due to the irregular changes in salinity, may be considered to reflect its ill-defined nature.

The environmental factors that brought about the seasonal difference in destalking effects thus appeared to be temperature and possibly light, but the contrasting results in the autumns of the two years were probably due to salinity. Unfortunately, due to the small number of experimental animals in (c) which included experiments under different lighting conditions, it was not possible to conclude the role light played. The positive effect on spawning of warm temperature occurring locally seems evident. The effect of lowering salinity on molting has been discussed; the effect of temperature will be described in the next section.

Effect of destalking on molting under different temperature conditions.

In the previous section, it was shown that spawning is restricted to the warmer months of the year. This section is only concerned with the effect of temperature on molting. If environmental factors in one season are more favorable than another it might be expected that destalked crabs will respond to the operation faster in the more favorable season. Results on three of the above experiments that provide data bearing on this aspect of the problem are compared in Table IX, in which time between destalking and molting is recorded. In the group of crabs destalked at the end of August, the average number of days before molting was

TABLE IX

The effect of destalking on molting under different temperature ranges

Crab no.	CW	Days between operation & molting	
4	54	19	
5	53	25	Average temperature: 28° C
6	71	42	Average CW: 72.0 mm
7	92	32	Average days: 32.4
8	72	23	Destalking month: August–September, 1966
9	73	49	Or: average CW: 78 mm
10	70	28	average days: 36 when nos. 4 and 5 are ignored
12	90	41	
F1	67	36	
F3	95	60	Average temperature: 23° C
F4	71	26	Average CW: 77.6 mm
F5	88	55	Average days: 44.8
F6	55	38	Destalking month: November, 1966
F8	77	54	
F9	87	44	
F10	80	47	
F11	72	53	
F12	74	35	
F17	94	51	
F19	75	33	Average temperature: 22° C
F23	82	43	Average CW: 77.5 mm
F24	88	82	Average days: 45.7
F26	59	28	Destalking month: March, 1967
F29	67	37	

36 for 6 crabs of average CW 78 mm (or 32.4 days for 8 crabs of average CW 72 mm, when 2 considerably smaller crabs are included). The average temperature was 28° C. In the group destalked in November, the average number of days was 44.8 for 10 crabs with average CW 77.6 mm. The average temperature was 23° C. In the group destalked in March, there were 6 crabs that responded by molting. Their average CW was 77.5 mm and the average number of days was 45.7. The average temperature was 22° C.

The salinity during the period of the above 3 experiments varied between 32–34 ‰ which was slight. Assuming that the morphogenetic effect of light was negligible after destalking, the only factor left for comparison among these three experiments was temperature, since salinity changes were not significant, and the same food was used in each experiment. The average size of the first group (when only the largest 6 crabs were considered) was very close to those of the second and third group.

The results indicated that the fastest response to destalking occurred at the highest temperature within the tested range and generally decreased as the temperature fell. In spite of the slightly larger size in the first group, the crabs molted faster. These data clarify the relationship between temperature and growth, the optimal conditions falling in the same month as ovarian development.

From this work, both molting and spawning should have their optima occurring in the warmest month. This month could become the peak of spawning but not molting, only if molting is inhibited by reproduction. Once this was established, the difference between the patterns of the histograms of molting and spawning in Figure 2 could easily be explained.

Jegla (1965) found in the cave crayfish *Orconectes pellucidus inermis,* living in nature under almost uniform temperature conditions throughout the year, that eyestalk removal was followed by molting more slowly in autumn than in summer. He suggested that an ecdysis had occurred in his crayfish in nature not long before they were collected for his autumn experiment. His reason why the autumn batch took longer to reach the subsequent ecdysis was thus attributed to their destalking earlier in the molt cycle than the same operation on the summer batch.

The present work shows that the number of days between destalking and molting increased from the first to the third experiment (Table IX), *i.e.,* from summer to winter, yet, the third experiment included un-molted control crabs left over from the second, and the second, those from the first. Obviously, no ecdysis had occurred in these crabs between experiments. Thus, it appeared to be the lowering of experimental temperatures, and not destalking earlier in the molt cycle, that lengthened the periods required for the crabs to molt.

Endocrine functions at post-molt

Second event after molting in destalked crabs In order to observe the second event (molting or spawning) after molting, destalked crabs were maintained in the laboratory as long as they survived. Results are given in Table X, which only includes crabs responding to the operation by molting. Crabs that died within a few days after molting were also excluded since their death was apparently not related to a second event. From the Table, crabs that were destalked in February and March and that responded by molting all spawned subsequently. Those

Table X

Second event after molting in destalked adult female stone carbs

No.	CW	Destalked	Molting	Second event	Date of event
4	54–72	Aug. 31, 1966	Sep. 19, 1966	Spawned few eggs	Oct. 11, 1966
5	53–73	Aug. 31, 1966	Sep. 25, 1966	Spawned few eggs	Oct. 18, 1966
8	72–94	Aug. 31, 1966	Sep. 23, 1966	Spawned few eggs	Oct. 18, 1966; Jan. 3 and Feb. 28, 1967
F4	71–90	Nov. 2, 1966	Nov. 28, 1966	Spawned few eggs	Jan. 18, 1967
F9	87–103	Nov. 2, 1966	Dec. 16, 1966	Spawned few eggs	Feb. 20; Apr. 11; May 20, 1967.
				Dead	May 21, 1967
F10	80–100	Nov. 2, 1966	Dec. 19, 1966	Spawned few eggs	Feb. 18; Apr. 9, 1967
				Dead	Apr. 30, 1967.
F11	72–92	Nov. 2, 1966	Dec. 25, 1966	Spawned few eggs	Mar. 28, 1967
				Dead with brittle shell	Apr. 30, 1967
F14	95–110	Nov. 2, 1966	Dec. 12, 1966	Spawned few eggs	Feb. 24, 1967
				Dead	Mar. 8, 1967
F1	67–84	Nov. 2, 1966	Dec. 8, 1966	Dead, ovary white	Feb. 3, 1967
F3	95–109	Nov. 2, 1966	Jan. 1, 1967	Dead, ovary resorbed	Jan. 25, 1967
F5	88–106	Nov. 2, 1966	Dec. 27, 1967	Dead, ovary resorbed	Mar. 10, 1967
F6	55–73	Nov. 2, 1966	Dec. 10, 1967	Dead, ovary undeveloped	Mar. 2, 1967
F7	85–109	Nov. 2, 1966	Nov. 28, 1967	Dead, with few mature eggs in oviduct	Apr. 14, 1967
F12	74–92	Nov. 2, 1966	Dec. 5, 1966	Dead, ovary resorbed shell brittle	Apr. 30, 1967
C	74–93	Feb. 8, 1967	Mar. 20, 1967	Spawned	Apr. 27, 1967
				Dead, ovary medium in size.	Jul. 7, 1967
10	86.5–104	Feb. 7, 1967	Mar. 30, 1967	Spawned	Apr. 30, 1967 Jun. 5, 1967 Aug. 22, 1967
F17	94–?	Mar. 24, 1967	May 14, 1967	Spawned	Jun. 16, 1967
				Dead	Jul. 17, 1967
F23	82–103	Mar. 24, 1967	May 6, 1967	Spawned	Jul. 27, 1967
				Dead	Jul. 28, 1967
F29	67–84	Mar. 24, 1967	Apr. 30, 1967	Spawned	May 28, 1967; Jul. 9, 1967 Jul. 28, 1967.

destalked in August or November either spawned or died subsequent to molting. Further, those that spawned produced fewer eggs than those operated in the spring. Among those that died, one contained a small number of mature eggs in the oviducal region, which could only happen if the crab had recently spawned. All the rest did not have a mature ovary.

The results suggest that while the crabs included in the Table molted following destalking, the operation also caused spawning after molting, even within the off-season when ovarian development rarely occurred.

Destalking of post-molt adult female crabs As post molt crabs were not easily available, only a few were employed in this experiment. In Table XI, 4 crabs were destalked 2 to 6 days after they had molted in the laboratory, while 2 others were operated on a little over one month after molting. All operated crabs responded by spawning, although they were destalked in the non-spawning season. Crab number 24 spawned only a small number of eggs because it was destalked in a period during which little egg development had taken place.

Conclusions In these experiments, both molted destalked crabs and destalked postmolt crabs spawned even in an environment under which egg development and spawning rarely took place (compare with Table III and IV for intermolt crabs). Although destalked crabs at postmolt did not appear to accelerate ovarian development, it is possible that crabs early in their molt cycle tended to spawn, while those reaching premolt stage showed a tendency to molt. This is not difficult to explain since newly molted crabs had probably passed their highest titer of molting hormone. At intermolt, the tendency depends on the ovarian development which in turn depends on seasonal factors (above).

Finally, it is interesting to note that spawning could take place in such crabs without a normal amount of mature eggs during off-season. This finding deserves further investigation, as it may be reflected by the existence in the eyestalks, of separate controlling mechanisms for spawning and egg development.

TABLE XI

Destalking experiment on adult female post-molt stone crabs in winter-spring

Crab no.	CW	Date molted	Destalked	Spawned	Post spawned molting	Dead
24	81	Oct. 26, 1966	Nov. 2, 1966	Dec. 27, 1966	—	Jan. 22, 1967
F30	62	Jan. 8, 1967	Jan. 11, 1967	Feb. 22, 1967	Apr. 23, 1967	Aug. 9, 1967
F31	100	Jan. 10, 1967	Jan. 13, 1967	Mar. 6, 1967	—	Apr. 30, 1967
F32	103.7	Dec. 4, 1966	Jan. 18, 1967	Mar. 7, 1967	—	
F33	81	Mar. 23, 1967	Mar. 25, 1967	Apr. 20, 1967	—	Apr. 30, 1967
23	115	Jan. 2, 1967	Feb. 7: 1967	Mar. 14, 1967	—	

DISCUSSION

Based on this work the adult female phase of the life cycle of *M. mercenaria* could be described as follows:

After the crab reaches maturity, the development of its ovary is probably initiated in nature during seasons of increased light intensity and warmer temperatures. Subsequent development seems favored by warm temperature alone. This may be the reason why the spawning peak did not coincide with the day-length peak. Because of the egg development, growth is inhibited, in spite of the fact that warmer temperatures also favor growth. After each spawning, the incubation of the eggs carried under the abdomen also inhibits molting. The mechanisms by which incubation inhibits molting are still unknown. The shift of season begins probably as the temperature drops in autumn. By this time new egg formation could be arrested, probably through diminishing light intensity. As soon as the latest batches are mature, spawned, and hatched after incubation in high summer, a new molting season begins in the fall. Even during the spawning season, an ecdysis could occur between two spawnings, whenever there is a lack of molt inhibition. It is likely that growth is possible all year around even in the coldest month, since the temperature in this tropical area rarely drops below the limit of molting blockage (Passano, 1960b). (The lowest monthly average from Figure 1 was slightly below 20° C, whereas from unpublished data on culturing this species, about 15° C has been found to be within the range of the blockage.) This would result in the molting season being distorted towards autumn when the temperature is less favorable. While reproduction and growth are antagonistic to each other, it is reproduction that inhibits molting; there is no evidence in any time of the year that molting could inhibit reproduction. In the pre-adult stages, *i.e.* juvenile and larval, when growth is the predominant activity, growth should proceed maximally in summer when the temperature is highest.

The alternation of dominance between molting and spawning is controlled by external factors as well as the molting cycle. While previous work on other species (Drach, 1944) demonstrated that once premolt was initiated the cycle proceeded to the molt, the present study suggests that at postmolt, spawning is dominant in adult female *Menippe,* irrespective of seasons. However, spawning and ovarian development are probably under separate control. During the intermolt stage, dominance depends also on external factors. Crabs within the intermolt stage could molt following destalking at one time, whereas, they could spawn if the operation is performed later in a different season. Obviously, this is determined by the state of ovarian development. Thus, along the change from postmolt via a long intermolt to premolt, crabs do not necessarily transfer irreversibly from the dominance of spawning to that of molting since, during the intermolt stage, the dominance of molting may be changed back to that of spawning if the ovaries mature.

As suggested in the above, at the spawning season, the process of ovarian development initially, at least, inhibits somatic growth. Later, after each spawning, the incubation process inhibits molting.

When one considers the well known effects of the endocrine system in a crab, it is possible that molting could be inhibited in one or more of the following ways: (a) an increase in circulatng MIH, (b) a decrease in concentration of OIH, (c) an increase in the effective concentration of ovarian promoting hormone from certain neurosecretory cells in the CNS, (Otsu, 1963) or (d) a decrease in secretion of molting hormone by the Y-organs (Démeusy, 1962).

If the regulation is entirely effected by (a) and (b) it is easy to imagine that any visual stimulus affecting MIH or OIH secretion would act through the eyes, as there should be some adaptive reason for the X-organs to be situated so near the eyes. This is most likely the case only when light intensity is concerned, but certainly not temperature, which acts through the whole organism. Also, what stimulates the MIH activity if the inhibition of molting is due to egg-bearing? If the egg-bearing pleopods receive their stimuli by way of the nervous system, would the stimuli not reach the thoracic ganglion and brain before they finally reach the X-organ? In this case, would it not be possible as well as simpler if inhibition could be effected by method (c)? If this is the case, a forward step is made towards the understanding of this problem since Scudamore (1948). It is not surprising that all the above four suggested processes could happen naturally. So far, the relation between the X-organ and Y-organ seems to be well established, that between the X-organ and the ovarian accelerating neurosecretory activities in the

CNS may also exist. A possible antagonistic relation between the Y-organ and the ovarian accelerating hormone-producing neurosecretory cells has not yet been investigated.

The estimate in the above that there were 4.5 spawnings to a molt in the average adult female crab has not taken into consideration the fact that the crabs were isolated. No male crabs were introduced for copulation at each molting. Although it has been proved (Cheung, 1968) that viable sperm could be retained after molting in the female stone crab, it is still uncertain whether a copulation with introduction of new sperm into the female would have any effect on ovarian development as well as on the frequency of spawning. This should certainly deserve future investigation.

Finally, I like to compare the biology of *M. mercenaria* with that of another crab, *Carcinus maenas* which can also molt after reaching maturity. In the latter species inhabiting waters in Western Europe, growth as well as egg development is blocked by low temperatures (Démeusy, 1964), so that the molting season is predominantly in summer when the temperature regimen is most favorable.

Destalking adult *Carcinus* has been found to cause only ovarian growth and not molting in any season (Cheung 1964, Démeusy 1965b). It was during the juvenile stage and the terminal anecdysis (Carlisle 1957) that it would respond to the operation by molting. These may not be specific differences only between *Carcinus* and *Menippe*; they may also distinguish similar crabs distributed over colder and warmer waters, respectively.

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SUMMARY

1. The effects of certain environmental and physiological factors in the growth and reproduction of adult female stone crabs, *Menippe mercenaria* (Say), were studied between April, 1965 and November, 1967 by employing a long-term rearing method. The crabs were kept isolated from one another in tanks supplied with constantly running sea water pumped almost directly from Biscayne Bay and were fed daily with pink shrimp meat.

2. Results showed that spawning was most frequent in warm temperature, indicating that the seasonal dependence of spawning may be related to seasonal temperatures. Molting occurred most frequently in autumn-winter, a period of decreased spawning, although summer temperature seemed more favorable to molting. Summer molting may therefore be inhibited by reproductive activities.

3. In order to study the control of molting and spawning, several experiments involving the destalking of crabs were performed in a 13-month period. Destalking in August and September of 1966 induced molting. After September, crabs responded to destalking either by molting or by spawning. As the year progressed, the proportion of spawning to molting crabs increased until in September, 1967

spawning was the only response. This finding not only supported the work on *Gecarcinus lateralis* (Weitzman, 1964) in that there was a cyclic change in the dominance of molting and spawning, but also indicated a transitional period between the periods when molting or spawning are dominant.

4. The effect of destalking postmolt crabs was studied. The results indicate these crabs spawn precociously, but do not undergo accelerated ovarian development. This indicates that spawning and ovarian development may be controlled by different hormones.

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SEASONAL CHANGES IN THE THYROID GLAND IN THE MALE COBRA, *NAJA NAJA* L.

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The activity of the reptilian thyroid gland as judged by its histological appearance seems to be largely dependent on seasonal temperature and the reproductive state of the animal (see review, Lynn, 1969). Although these conclusions derive mainly from work on lizards, there is some information available on snakes. St. Girons and Duguy (1962) found that in two species of *Vipera,* the thyroid epithelial height is lowest in winter and increases to a maximum in early spring following the time of emergence from hibernation. It is low during the summer, but increases in autumn to a second peak. However, Binyon and Twigg (1965) found that *Natrix* presents a different picture from that of *Vipera.* In *Natrix,* the thyroid cell height is greatest in February, decreases steadily in July and October and is still low the following January.

In the female squamate, certain aspects of reproduction (*i.e.,* yolk deposition, ovulation and control of gestation in viviparous forms) are associated with thyroid activity (Eggert, 1935; Miller, 1955; St. Girons and Duguy, 1962; Wilhoft, 1964). In both sexes, the thyroid appears to be associated with periodic skin-shedding (see reviews by Sembrat and Drzewicki, 1936; Goslar, 1958; Maderson, 1965a; Chiu, Phillips and Maderson, 1967; Lynn, 1969). Both of these aspects of squamate biology should therefore be taken into consideration in any study of the seasonal variation in the thyroid gland.

In the present work, seasonal variation in the thyroid histology of the male cobra (*Naja naja* L.) has been studied, and an attempt has been made to demonstrate the extent to which the picture of annual thyroid activity might be affected by changes in the gland associated with the sloughing cycle.

MATERIALS AND METHODS

Each month from October, 1965, through September, 1966, 5 to 7 male cobras (*Naja naja* L.) from South China were obtained via local Hong Kong sources. After decapitation, the total body weight, and the weight of the abdominal fat body were recorded. A piece of the belly-skin was prepared for histological examination and the stage of the sloughing cycle established (Maderson, 1965b).

The thyroid gland was freed from connective tissue, weighed, and fixed in Bouin's fluid for 48 hours. It was then dehydrated, cleared in chloroform, and embedded in paraffin. Serial sections through the center of the gland were cut at 7 μ, mounted and stained with hematoxylin and eosin.

The assessment of follicular cell-height was made as follows. The 15 to 25 follicles which fell along the longest axis of the gland were examined. The tallest and shortest cell in each follicle was measured with an ocular micrometer. The average of these two values was taken as the cell-height for the particular follicle, and the mean of the cell-height for 15–25 follicles was taken as the cell-height for the gland. The average of the cell-heights of all glands examined during one month was taken as the cell-height for the month.

RESULTS

The thyroid weight as expressed on a fat free body weight was very low (11 mg%) in November prior to hibernation (Table I and Fig. 1). There was a significant increase in the weight of the gland during hibernation so that the February figure was 50% above the November figure ($P < 0.05$). Between February and June there was a steady decrease in weight (50%, $P < 0.05$). There was a rapid and significant increase (40%, $P < 0.01$) from June to July, followed by a steady decrease during the successive months August through October (see Fig. 1).

Changes in the cell-height of the gland do not correspond to weight changes (Table I and Fig. 1). In fact, when the gland weighed least in November and June, the follicular epithelium was high. There was no significant change in

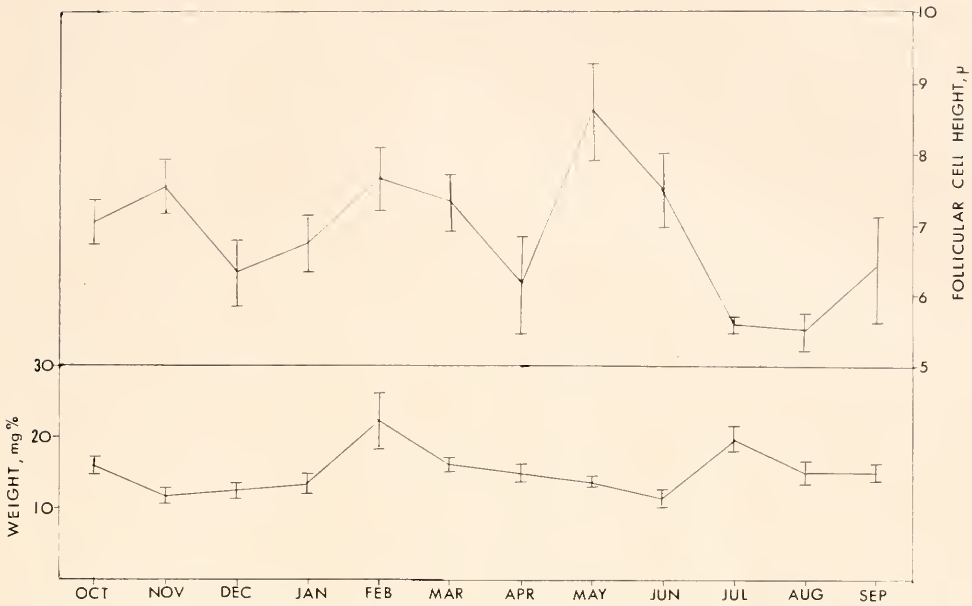
TABLE I

The weight and epithelial cell-height of the thyroid gland of the male cobra, Naja naja *L. in different months of the year*
Mean ± S.E.

Month of the year	No. of animal	Thyroid gland	
		Weight mg %$^{+}$	Cell-height μ
October, 1965	5	15.58 ± 1.70	7.06 ± 0.32#
November, 1965	6	11.79 ± 1.08*	7.54 ± 0.38
December, 1965	6	12.68 ± 1.17	6.33 ± 0.46
January, 1966	6	13.59 ± 1.49	6.75 ± 0.40
February, 1966	6	22.37 ± 3.82***	7.66 ± 0.45
March, 1966	6	16.24 ± 1.00	7.32 ± 0.40
April, 1966	6	14.96 ± 1.12	6.15 ± 0.70
May, 1966	7	13.84 ± 0.51	8.62 ± 0.71###
June, 1966	6	11.67 ± 1.28****	7.50 ± 0.52##
July, 1966	6	19.92 ± 1.96**	5.58 ± 0.11
August, 1966	6	15.16 ± 1.61	5.49 ± 0.27
September, 1966	6	15.16 ± 1.33	6.40 ± 0.75

$^{+}$ Fat-free body weight.
* Jul. v Nov. $P < 0.01$.
** Jul. v Jun. $P < 0.01$.
*** Nov. v Feb. $P < 0.05$.
**** Jun. v Feb. $P < 0.05$.
# Oct. v Aug. $P < 0.01$.
May v Apr. $P < 0.05$.
July v Jun. $P < 0.01$.

FIGURE 1



SEASONAL VARIATION IN THYROID EPITHELIUM, AND THYROID WEIGHT OF MALE COBRA, NAJA NAJA L. Vertical bars represent S.E.

follicular height during the hibernation period from November to April, but a 30% increase in May as compared to April ($P < 0.05$) suggests an increase in secretory activity. This increase was of short duration, being followed by a 35% decrease by July ($P < 0.01$), the cell-height showing an absolute minimum of 5.5 μ during August (compared with 8.6 μ in May). A steady increase in cell-height in subsequent months giving a 30% increase ($P < 0.05$) for October as compared to August, suggests an increase in gland activity following the inactive period during the summer.

The stage of the epidermal sloughing cycle of each animal used in the study is shown in Table II. As the cobra shows some variation in the degree of development of the alpha-layer at the time of shedding [*cf.* the lizard *Anolis carolinensis* (Maderson and Licht, 1967)], the staging is slightly different from that ascribed to *Elaphe taeniura* (Maderson, 1965b). For this reason, Table II shows no "stage 6" conditions as described by Maderson (1965b), but includes a readily recognizable "post-slough" condition when the alpha-layer is still being completed (Maderson and Licht, 1967); this is designated as Stage O. The distribution of epidermal conditions denoting the latter part of the renewal phase (Maderson, 1967) prior to shedding is of particular importance. Stages 4 and 5 (Maderson, 1965b) are represented by 0%, 33% and 33% of the animals sampled during August, July and April (the months during which thyroid epithelial height is lowest) and by 0%, 0% and 17% of the animals sampled during May, February and June (the months during which the thyroid epithelial height is greatest).

TABLE II

Stages of the skin and thyroid epithelial cell-heights in individual snake killed at each month

Month	Snake No.	Skin stage	Cell height μ	Month	Snake No.	Skin stage	Cell height μ
Oct.	151	5	8.15	Apr.	187	0	5.27
	152	1	6.46		188	5	6.42
	153	0	6.90		189	2	7.73
	155	5	7.28		190	3	4.74
	156	1	6.53		191	0	5.82
					192	5	6.91
Nov.	157	1	6.94		193	1	8.81
	158	1	8.53		194	2	10.85
	159	1	7.16		195	1	6.96
	160	5	6.80		196	2	7.12
	161	3	7.54		197	3	7.83
	162	1	7.74		198	3	7.27
					199	1	11.53
Dec.	163	0	5.72	Jun.	200	0	5.86
	164	0	5.66		201	1	6.95
	165	1	5.94		202	0	8.89
	166	0	8.62		203	5	8.84
	167	0	5.83		204	1	8.05
	168	0	6.24		205	0	6.41
Jan.	169	1	6.17	Jul.	206	1	5.86
	170	1	6.45		207	0	5.68
	171	0	6.80		208	2	5.46
	172	5	6.24		209	4	5.26
	173	0	8.68		210	1	5.33
	174	1	6.13		211	4	5.87
Feb.	175	1	7.68	Aug.	212	1	5.34
	176	1	7.94		213	0	5.19
	177	1	7.24		214	1	5.02
	178	1	10.77		215	2	5.31
	179	1	6.39		216	0	6.82
	180	1	5.93		217	2	5.26
Mar.	181	3	7.39	Sep.	218	0	5.75
	182	3	6.17		219	5	9.86
	183	1	8.06		220	1	6.11
	184	1	8.81		221	4	5.70
	185	1	6.95		222	4	6.56
	186	2	6.54		223	1	4.45

DISCUSSION

It has been shown elsewhere (Maderson, Chiu and Phillips, 1969) that in snakes, thyroid activity as judged by epithelial height, increases during the latter part of the epidermal renewal phase [stages 4–5 (Table II) and (Maderson, 1965b)] and decreases sharply during the resting phase, reaching its lowest point at the beginning of the new renewal phase [stages 2–3 (Table II) and (Maderson, 1965b)]. In the present context therefore, it is important to establish whether fluctuations in the pattern of thyroid activity are related to the sloughing cycle. We suggest that the distribution of epidermal conditions representing late renewal phase (stages 4–5) as shown in Table II is not correlated with the overall pattern of thyroid activity.

The determinations of thyroid weight indicate that there is a storage phase during hibernation, a release phase in the spring, followed by a rapid storage of secretion during the summer and another period of release in autumn. The pattern of secretory activity as judged by the follicular cell-height at different times of the year is in close agreement.

The present data show that there are two peaks of thyroid activity during the year in the cobra. The first is in May just after the animal emerges from hibernation, the second is during October-November just before the animal enters hibernation. This conclusion agrees with that of St. Girons and Duguy (1962) who reported on two species of *Vipera,* but is at variance with that of Binyon and Twigg (1965) for *Natrix*. Although Binyon and Twigg's work lacks statistical analysis, their data do suggest a slight increase in epithelial cell-height (about 25%) from October to January, and this might represent the second "peak" reported for *Naja* and *Vipera.*

As *Vipera* mates twice a year, in spring when the animal emerges from hibernation, and prior to hibernation in autumn, St. Girons and Duguy (1962) concluded that the seasonal changes in gland activity are associated with mating in the male, and ovulation in the female, rather than with environmental temperature. In fact, in both sexes of *V. aspis* and *V. berus,* low thyroid activity was noted during the period of highest environmental temperature, July and August.

Lofts, Phillips and Tam (1966) showed that after emergence from hibernation in April-May, the male cobra shows spermeiogenesis and mating. As this coincides with high thyroid activity, support is found for St. Girons and Duguy's (1962) conclusions for *Vipera.* However, as cobra only mates once a year, the second peak of thyroid activity in the autumn cannot be related to sexual activity as has been concluded for *Vipera* (St. Girons and Duguy, 1962) and some lizard species (Oliver, 1955; Wilhoft, 1964). Wilhoft (1963a, b, 1964) concluded that mating activity is not related to thyroid changes in the tropical skink, *Leiolopisma.* Data relating thyroid activity to spermatogenesis in squamates is paradoxical. Spermatogenesis in the fall has been reported in *Xantusia* (Miller, 1955) and three iguanid genera (Hahn, 1964; Licht, 1966; Wilhoft and Quay, 1961). In *Xantusia* (Miller, 1955), spermatogenesis in the fall is accompanied by high thyroid activity, but this is not true for *Sceloporus* (Wilhoft, 1958; Wilhoft and Quay, 1961). In *Leiolopisma* (Wilhoft, 1963a, b, 1964) and *V. aspis* and *V. berus* (Volsøe, 1944; St. Girons, 1957; St. Girons and Duguy, 1962) where spermatogenesis is continuous throughout the year, it is not correlated with thyroid activity. However, the present data show that high thyroid activity in the cobra is correlated with testicular recrudescence in September and spermiogenesis in the following spring as reported by Lofts *et al.* (1966). In fact, these authors (Tam, Phillips and Lofts, 1967) later show two peaks of testosterone production from the male gonad which are coincident with the peaks of thyroid activity described here, but in the absence of further information on seasonal variation in androgen production in other squamates, no general conclusion regarding the relationship between the thyroid and the testis is possible.

There is a considerable body of literature relating thyroid gland activity to environmental temperature in poikilotherms (Lynn, 1969). Investigations of natural and experimental populations of lizards have shown that high temperatures are associated with high thyroid activity. However, according to the results presented here for *Naja naja,* for *Vipera* (St. Girons and Duguy, 1962) and for *Natrix* (Binyon and Twigg, 1965), snakes appear to show low thyroid activity during the summer months. To what extent this reflects fundamental differences in thyroid function between the two squamate groups, and/or behavioral mechanisms associated with thermo-regulation (see review, Brattstrom, 1965) must await further data.

Three points merit consideration as possible causes of seasonal changes in snake thyroid function. First, in lizards which remain active during winter, thyroid activity remains high, but in hibernating forms, the activity is lower at this time (see review, Lynn, 1969). Tropical forms show no significant seasonal variation (Wilhoft, 1963a, b, 1964). Second, low thyroid activity in snakes during the summer months need not necessarily be associated with the environmental temperature if the animal shows thermo-regulatory behavior (Brattstrom, 1965; Hutchison, Dowling and Vinegar, 1966). Third, the present study cannot support St. Girons and Duguy's (1962) conclusion that peaks of thyroid activity in snakes are associated with mating behavior. We conclude that increase in thyroid activity in snakes is associated with the animal's activity, both behavioral and metabolic. Thus the post-hibernation peak of thyroid activity could be associated with a variety of behavioral activities, *e.g.,* food-finding, territorial establishment and also mating. Intensive food-searching (leading to a storage of fat utilized during hibernation) in the autumnal pre-hibernation period, would possibly represent a total increase in general activity. That autumnal food-searching leads to fat storage before hibernation is suggested by the fact that hibernating animals show 10% body weight represented as fat during March, against only 0.1% during July (Chiu, unpublished data).

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SUMMARY

1. Variations in the weight and follicular cell-height of the thyroid gland of the male cobra (*Naja naja* L.) have been studied for 12 consecutive months during a one year period.

2. Changes in the weight of the gland were not correlated with follicular cell height.

3. The epithelial height was greatest during May and October-November, lowest in August.

4. Variations in the thyroid gland activity that are associated with epidermal sloughing are not great enough to alter the overall pattern of seasonal variation.

5. It is suggested that the annual cyclic changes in the thyroid gland activity are probably related to the general activity of the male animal throughout the year and are not associated with fluctuations in environmental temperature nor, as in the female, with any specific aspect of the reproductive cycle.

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STUDIES ON THE BEHAVIOR OF *NASSARIUS OBSOLETUS* (SAY) (MOLLUSCA, GASTROPODA)

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Nassarius obsoletus (Say), the American mud snail, is an active and abundant prosobranch on the east coast of the United States of America. It is commonest on muddy shores where streams render the water brackish.

C. E. Jenner, in a series of short abstracts (1956, 1957, 1958 and 1959) has described the way in which up to several thousand of these animals may move in contact with each other over the mud flats at Barnstable, Cape Cod. Such schooling was also mentioned in Dimon's monograph (1905), although she seems to have seen it only rarely at Cold Spring Harbor. Schooling is most apparent when the snails are submerged. With exposure to the air at low tide many of the members of a school bury whilst others continue schooling. Physical contact plays an important role in promoting uniform orientation within a school, and Jenner (1957, 1958) suggests that vision and chemical factors may also be involved. He asserts the importance of water currents in determining the direction of movement of a school, which is in general with or against the stream, although schools were observed sometimes to move across the current and where there was no current.

Jenner (1956, 1957, 1958, 1959) also described a change in the distribution of the populations of *Nassarius* from wide dispersal over the mud banks in the harbor to aggregation into dense clumps two to three individuals deep. The change was observed in four consecutive years, from 1956 to 1959, and took place in late July or early August, towards the end of the period of reproductive activity. An autumn migration of populations of *Nassarius* from the littoral to the sub-littoral has been described by C. H. Batcheldor (1915) and by C. J. Sinderman (1960). During the migration snails move in schools, and accumulate in dense aggregations, often covered by sediment below the level of low tide. There they overwinter in a quiescent state, returning to the mud flats in spring.

The present study of *Nassarius* was undertaken in the hope that laboratory reactions to controlled stimuli could be correlated with the behavior of individuals and schools in the field. Dimon's monograph on *Nassarius obsoletus* included experiments on the reactions of small numbers of individuals to light, gravity and water currents. Her conclusions are not always in agreement with mine. Copeland in 1918 described the positive rheotaxis of *Nassarius* when stimulated by *Fundulus* juices. His study was confined to olfactory stimuli.

OBSERVATIONS

Movements of Nassarius obsoletus *in Barnstable Harbor*

The snails were observed on five occasions on the extensive mud-sand flats of Barnstable which are exposed only at low tide. *Spartina alterniflora* grows on the higher banks and is also covered at high tide.

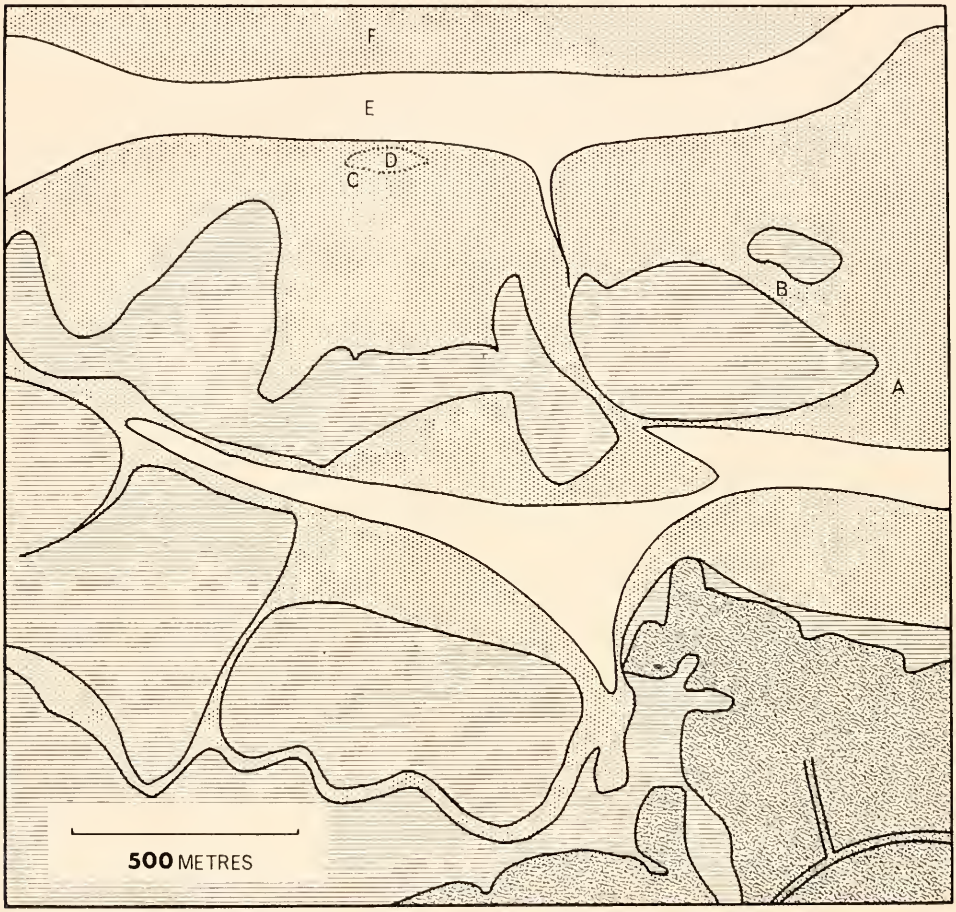

FIGURE 1. Sketch Map of *Nassarius* Area

A. Source of diatomaceous mud.
B. Source of purple bacteria mud.
C. Shallow stream.
D. Small bank.
E. Main stream.
F. Main bank.
Heavy stipple indicates land.
Horizontal hatching indicates marsh.
Dotted stipple indicates sandy mud banks exposed at low tide.

On 14th July, 1967, during a falling spring tide there were large numbers of *Nassarius* of 1½–2 cm. shell height on the small sand bank shown in the sketch map (Fig. 1). Schools of variable size, often consisting of several thousand individuals moving together and forming a continuous monolayer of snails, were to be seen on the damp surface of the sand bank and just below water level. There were also many individuals wandering freely, not attached to schools. Schools moved in all compass directions, usually downhill in the direction of drainage. Congregations of schools were to be found in pools on the sandbank and at the margins of the bank. When an artificial pool was dug and began to fill with water, many mud snails entered it from the surrounding area in the same way that they entered natural pools. Drainage of a natural pool by digging a channel in the mud resulted in increased activity in the pool with a general tendency of the animals to move downhill. Some animals found the channel and moved down it.

To test the importance of draining water for movement of *Nassarius,* some 40–50 individuals were placed on a dry sloping part of the bank and water was poured over them. They moved downhill into the main stream. Another group placed in a similar position, but not drained with water, stayed still. When the bank became dry, schooling stopped, leaving most of the animals in damp hollows where they partly or completely buried.

As schools reached the edge of the main channel and became submerged the tendency was to move upstream, although exceptions were observed. A typical count taken under 15 cm of water where there was a strong current showed 106 individuals moving upstream to 4 moving down. In the shallow stream a sample had 60 moving up to 1 moving down. Single animals from deeper in the main channel moved uphill at right angles to the current.

On 25th July, 1967, the same bank was observed on an incoming tide. Specimens of *Nassarius* were to be seen in the hollows on the bank and schooling in the surrounding channels. The underwater schools tended to move uphill towards the waterline, and were less closely packed than those on the bank.

On 2nd August, 1967, the bank previously observed was supporting a bloom of purple bacteria and few specimens of *Nassarius* were to be seen. Small numbers were present higher up the shore, moving upstream in runnels draining from the marsh.

On 7th August, 1967, members of the species *Nassarius* were still lacking from the small bank but were abundant higher up the shore, forming a wide band from the shallow stream towards the marsh. The animals seemed generally smaller than the population seen before and included a size group of 4–5 mm shell height not previously noticed. Large numbers of *Nassarius* were present at High Water Neaps level to Mid Tide level among the lowest clumps of *Spartina.* The main banks were more heavily populated than before. On the main banks the snails were concentrated in pools or damper areas whilst firmer clean sandy areas of up to 20 square meters were often free of snails. No orientations were visible as the animals had been exposed for some time so that many had buried.

On 17th August, 1967, mud snails were back in their former abundance on the small bank and were present in vast numbers higher up the shore. In the marsh area small individuals were massed in the upper reaches of drainage runnels in seething aggregations several individuals deep. The aggregations may be similar to those described by Jenner (1956, 1957, 1958, 1959), although the animals he

Table I

Behavior of transplanted Nassarius

Movement of snails with respect to current		Behavior of transplanted snails		
At source of transplanted snails	In area to which transferred	No. moving upstream	No. moving downstream	% UP
A) At low water mark				
Up	Down	27	23	54
Down	Up	21	8	72
Down	Main channel (no native snails)	Random. Tendency to move uphill to waters' edge.		
B) On the marsh				
Up	Down	39	5	89
Down	Up	23	16	59
Down	Down (control)	0	8	0

observed were post-reproductive, whereas the 4–5 mm individuals were the result of the 1967 reproductive activity and not yet themselves of an age to reproduce (see Scheltema 1964). In the marsh areas strong upstream migrations were to be seen, but in the highest reaches some downward migrations occurred. The migrating schools kept close to the interface between mud and water and avoided deeper parts of the channels, walking up the slope to the edge. Where animals moving in opposed directions met, aggregation occurred.

Experiments were performed to see whether the movement of snails upstream in runnels draining off the marsh was a result of conditions in the water or of the physiological state of the animals. Snails were removed from an area in which movement was predominantly upstream and placed in a stream where the local inhabitants had been moving downstream but from which they were removed. The converse experiment was tried for downstreaming snails. The results are shown in Table I.

A proportion of upstreaming animals, especially small ones from the marsh, continued to move upstream in a downstreaming area. Animals taken from an area where the predominant movement was downstream accommodated more readily to moving upstream in an area where others had been doing so. The condition of the animal as well as that of the water seems important in determining the direction taken in a stream. Once the main convoys of snails came into contact with the transferred group the former quickly established dominance, the transplanted animals conforming to the general direction after a brief struggle and blockage.

Mutual conformity

When a *Nassarius* is placed in a dish of seawater and left undisturbed for a few minutes, it goes to the edge of the dish and moves round it in a clockwise or anticlockwise direction. Snails had a greater tendency to turn right at the edge of a dish and move clockwise than to turn left, whether in daylight or total darkness. This tendency was noted also by Dimon, and is probably a consequence of the asymmetry of the animal's gross morphology. Schooling requires the individual to conform with the direction of movement of the group. It is a simple matter to induce a number of snails to follow one another round the edge of a dish, forming a model of schooling behavior.

When another snail was placed in the center, it moved to the edge and was often seen to turn smoothly and follow the direction of movement of those already in procession. Such "invaders" attempting to enter the column in the wrong direction, an event which happened rarely, were soon nudged into conformity or, very rarely indeed, caused all movement in the dish to stop. The results of an experiment in which individuals were introduced singly into a dish containing 7–10 processing snails, are shown in Table II. There is a highly significant tendency for the invader to conform to the direction of movement of those already present, a tendency which may have a bearing on schooling behavior.

TABLE II

Effect of a convoy on snails entering

Direction of movement of convoy	Direction of turning of introduced snail	
	Clockwise	Anticlockwise
Clockwise	25	1
Anticlockwise	1	25
Control (no convoy)	30	22

Not only did an established column of snails influence the turning of newcomers, but the impression was gained that unanimity of direction was more often attained by a group of snails starting simultaneously from the center of a dish than would be expected from the behavior of isolated individuals. Sets of from 2 to 10 individuals were placed in a fingerbowl 17 cm in diameter, filled to a depth of 2–3 cm with seawater. After six minutes the orientation of all the animals lying along the edge of the dish was recorded as clockwise or anticlockwise. Even with the larger numbers in a set it often happened that all were orientated in the same sense. These sets were referrd to as "unanimous," either clockwise or anticlockwise. When a single individual failed to conform with the rest of the set, the result was classified as "all but one conforming," and when more than one individual failed to conform, as "residual." Table III gives the number of sets of animals falling into these categories.

In order to determine whether one individual influences another, it is necessary to compare the results given in Table III with the distribution of orientations to be expected if each individual behaved independently. If p is the probability of turning clockwise and q the probability of turning anticlockwise, then since these are the only possibilities allowed for in the experiment, $p + q = 1$. According to Dimon (1905) this species has a greater tendency to turn right than to turn left; it cannot therefore be assumed that $p = q = \frac{1}{2}$. A large number of trials was made

TABLE III

Effect of number of snails in a dish on conformity

		Number of sets that were:				
I No. of snails per dish	II No. of sets tested	III Unanimous clockwise $m = s$	IV Unanimous anticlockwise $m = 0$	V	VI	VII Residual
				All but one conforming		
				Clockwise $m = s - 1$	Anticlockwise $m = 1$	
Set of 2	48	$\underline{\underline{31}}$ (21.3)	$\underline{\underline{10}}$ (5.3)	7 (21.3)		x
Set of 3	27	$\underline{\underline{11}}$ (8.0)	1 (1.0)	$\underline{10}$ (12.0)	$\underline{5}$ (6.0)	x
Set of 4	27	$\underline{\underline{10}}$ (5.3)	0 (0.3)	$\underline{6}$ (10.7)	$\underline{\underline{5}}$ (2.7)	$\underline{6}$ (8.0)
Set of 5	20	$\underline{\underline{6}}$ (2.6)	$\underline{\underline{1}}$ (0.1)	$\underline{2}$ (6.6)	$\underline{\underline{2}}$ (0.8)	$\underline{9}$ (9.9)
Set of 6	20	$\underline{\underline{8}}$ (1.8)	$\underline{\underline{2}}$ (0.0)	$\underline{4}$ (5.3)	0 (0.3)	$\underline{6}$ (12.6)
Set of 7	19	$\underline{\underline{5}}$ (1.1)	$\underline{\underline{1}}$ (0.0)	$\underline{\underline{4}}$ (3.9)	0 (0.1)	$\underline{9}$ (13.9)
Set of 8	25	$\underline{\underline{4}}$ (0.9)	0 (0.0)	$\underline{3}$ (3.9)	$\underline{\underline{3}}$ (0.1)	$\underline{15}$ (20.1)
Set of 9	13	$\underline{\underline{6}}$ (0.3)	0 (0.0)	$\underline{\underline{4}}$ (1.5)	$\underline{\underline{1}}$ (0.0)	$\underline{2}$ (11.1)
Set of 10	10	$\underline{\underline{2}}$ (0.1)	0 (0.0)	$\underline{\underline{5}}$ (0.5)	0 (0.0)	$\underline{3}$ (9.5)

Values obtained from binomial distribution in parentheses. Values greater than expectation underlined twice. Values less than expectation underlined once.

x indicates impossible situation.

with single individuals to determine how many turned right or left and so to assign values to p and q. Of nearly 300 individuals exactly twice as many turned right as turned left. The same tendency was noted in many of the later experiments and could also be demonstrated in total darkness.

The probability of m out of a set of s individuals in the same dish turning in a clockwise sense will be given by the formula :—

$$Pm = \frac{s!}{m!\,(s-m)!}\,p^m\,q^{s-m}$$

if each individual behaves independently.

The above formula was used to obtain values of Pm for $m = s$; $m = 0$ (unanimous situation) and for $m = s - 1$; $m = 1$ (all but one conforming) with $p = 0.667$, $q = 0.333$. By multiplying the calculated values of Pm by the total number of sets (column II), the theoretical number of sets of each category can be calculated. These values are shown in parentheses next to the observed numbers. The table shows that the observed number of sets in which all the snails conformed was much greater than expectation (columns III and IV), while the number in which more than one snail failed to conform (column VII) was less than expectation. A χ^2 test of grouped data indicated a significance level of $P < 0.001$.

TABLE IV

Effect of "artificial" snails on degree of conformity

Invading snails entering a procession of	No. of invading snails moving		Total
	With procession	Against procession	
Normal living *Nassarius*	80	2	82
Wax-covered live *Nassarius*	38	2	40
Wax-covered empty shells	67	23	90

It might be thought that a snail entering a convoy conformed with its direction of movement merely because it was pushed. Observation of the entering snails did not support this theory, since snails usually glided in unobtrusively, rarely blocking or barging into the column. An attempt was made to analyze the sensory cues enabling a snail to follow the direction of movement of a convoy.

Tactile responses

A series of experiments was performed using empty shells of *Nassarius*, cleaned of algae by boiling in caustic soda and completely covered in paraffin wax, which were suspended in a dish by wires attached to a rotating kymograph drum. The "artificial snails" could thus be moved in convoy at a speed comparable to that of a procession of real snails. The shells were arranged so that they faced forwards when moved anticlockwise. Test snails were placed in the center of the dish and their reactions upon reaching the procession of real or artificial snails at the edge was recorded, noting also, if they conformed to the direction of movement of the column, whether they did so with or without being pushed. No significant difference in behavior was detected between entry into clockwise columns and into anticlockwise columns. Results from the two are therefore not distinguished in Table IV.

Invading snails are seen to conform equally readily to the direction of movement of wax-covered and normal living snails. The coat of wax cannot therefore be responsible for the significantly reduced success of the artificially moved snails in inducing others to conform. The proportion of snails forced into conformity by pushing was higher when artificial snails were used, as shown in Table V.

Artificial snails lack a foot, which may, by touching that of the invading snail, persuade it round; they do not lay a mucous trail; and they might not emit the same scent as real snails.

TABLE V

Effect of "artificial" snails on manner of achievement of conformity

Processing snails	No. of test snails conforming	
	Without push	With push
Normal live *Nassarius*	27	3
Waxed live *Nassarius*	30	8
Waxed shells	29	38

The possible importance of the mucous trail is demonstrated by a series of experiments in which snails were allowed to process round a dish for 15–20 minutes and were then removed before test snails were introduced singly. The first series was conducted in full knowledge of the sense in which the dishes had been conditioned; in the second series the snails were introduced without foreknowledge of the direction in which the dishes had been conditioned. The two series produced almost identical results, shown in Table VI. It seems that on glass *Nassarius* is able to respond to the direction in which other mud snails have recently been moving. The obvious interpretation is that a mucous trail has been laid down which is polarized in some way that is recognizable to the snail. No visible directionality could be discerned when trails were stained with Methylene blue or Alcian blue and examined under the microscope. Nor could the effect be observed when there was no obstacle corresponding to the edge of the dish against which the snails would turn. A glass ring was temporarily used to confine a procession of snails, and removed. Snails entering the conditioned area did not follow the mucous trail.

Table VI

Effect of pre-conditioning a dish

Conditioning of dish	No. of animals turning	
	Clockwise	Anticlockwise
Clockwise	38 + 44* = 82	8 + 16* = 24
Anticlockwise	16 + 20* = 36	29 + 40* = 69
Controls (dishes not conditioned)	34	17

* Second blind series.

It proved impossible to repeat the experiment on a more natural surface of mud since snails put in to condition the mud ploughed it up and often buried. The significance of conditioning of surfaces by snails in the natural habitat is thus unknown. It is however most important when performing experiments on glass surfaces in the laboratory to obviate any possible interference by mucous trails.

Visual responses

The reaction of *Nassarius obsoleta* to a bright light is variable, and this may account for reports that the animal does not respond to light. However, the animal reacts very clearly to a shadow, although the response soon habituates.

In order to decide whether vision could play a part in schooling or navigation, the animals' response to moving patterns (optomotor response) and to light sources was investigated. The usual type of apparatus involving rotation of a glass dish containing the animal or of a cylindrical pattern around the animal, was employed. The dish was cleared of mucous trails between trials. The results of the experiments are shown in Table VII. In both experiments involving a light compass stimulus the animals tended to maintain station; when the light source was unobstructed (experiment 1) the effect was highly significant, though the animals achieved a constant angle to the light for only short periods of time. The possibility that water currents induced by inertia produced the orientation was eliminated by experiment 3 in which, without a directional light source, no light compass response was given.

The failure of *Nassarius* to follow an optomotor drum with stripes each subtending as large an angle as 60° at the center, renders it most unlikely that behavior leading to schooling involves visual recognition at a distance of other snails and their direction of movement. The light compass reaction may account for the persistence of orientation of schools or individuals when other stimuli seem inconstant or lacking.

TABLE VII

Visual responses

Experiment	Condition	Illumination	Cylinder	Stimulus given	Average rotation to clockwise stimulus	t test	Average rotation to anticlockwise stimulus	t test
1	Moving dish	Side	None	Light compass	+1.08	$P=0.005$	+0.55	$P=0.001$
2	Moving dish	Side	Plain white	Light compass (weak)	+0.21	N.S.	+0.95	N.S.
3	Moving dish	Above	Plain white	None	−0.01	N.S.	−0.04	N.S.
4	Moving dish	Above	60° alternating black and white stripes	Optomotor	−0.04	N.S.	+0.01	N.S.
5	Moving cylinder	Above	60° alternating black and white stripes	Optomotor (no water movement)	−0.20	N.S.	+0.08	N.S.

Values give the number of complete turns (2π) during movement from center to edge of dish. Positive sign implies maintaining station with reference to the rotation of light or pattern.

Rheotactic behavior

Draining slopes On the exposed mud flats, mud snails were observed to move predominantly downhill. This reaction was imitated in the laboratory. A gentle flow of seawater was allowed to run down a plane of glass sheet inclined at a small angle to the horizontal. Batches of specimens of *Nassarius* were placed about two-thirds of the way up the slope, facing in random directions. Direction of movement up or down was noted when individuals had reached the top or bottom of the slope; it was predominantly downhill. After some trials the glass slope was covered with absorbent paper to ensure that movement of the snails down the slope was not due to passive sliding. The result was the same. A wooden slope also elicited the same reaction. A lamp placed near the uphill or downhill end of the slope did not affect results. Predominantly downhill movement was also observed on damp sloping paper with no water flow. Under water the direction of movement on a slope was not consistently up- or downhill being much affected by light, as shown in Table VIII. On the vertical glass sides of the stock tank animals newly put in tended to climb upwards. The same reaction was noted by Dimon who also claimed that *Nassarius* was negatively geotropic in air, contrary to the present findings.

Water currents To test the behavior of *Nassarius* towards a water current in the laboratory, a gentle flow was set up in a fingerbowl of diameter 17 cm filled to a depth of 2–3 cm with seawater, by directing a jet of compressed air along the water surface near one edge of the bowl. The bowl was screened from lateral light by a ring of opaque card and was thoroughly cleaned between trials. Animals were placed singly in the center of the dish, and their reactions recorded in one of two ways. In the first series of experiments, the directions of the initial turn on emerging from the shell, and of the final turn on reaching the edge of the dish were noted. In the second series, using smaller dishes (diameter 10 cm) the total turning of the animal was algebraically summed in units of 45°, clockwise turning being taken as positive, anticlockwise as negative.

TABLE VIII

Effect of slopes

	No. of animals moving	
	Up	Down
1. *In Air*		
With water flow		
Glass slope	12	36
Paper slope	9	143
Wooden slope	2	21
Without water flow		
Damp paper	4	35
2. *In Water*		
Light at upper end	28	4
Light at lower end	7	13

The first series of experiments showed that although the initial turn is not significantly affected by current direction (χ^2 test), the final turn shows a marked tendency of the animals to move with the current. (χ^2 test $P = 0.001$). This was confirmed by the second method which was used in subsequent experiments.

Influence of olfactory stimulants The addition of substances derived from injured animal tissues to water circulating in bowls and to draining slopes causes a reversal of rheotaxis from negative to positive, as described by Copeland (1918). An exactly similar reversal of movement can be obtained when olfactory stimulants are added to water draining down slopes. In most experiments the same individuals were used first as controls in clean seawater and subsequently for experiments with stimulating substances. Positive responses sufficiently clear to establish approximate thresholds were consistently obtained only from snails starved for at least 48 hours in the laboratory. As shown in Table IX, *Carcinus* extract, prepared by crushing a fresh specimen in 10 cc of seawater and filtering the resulting fluid was effective at a concentration of 3×10^{-2} or more in seawater, and so was *Libinia* blood at 1×10^{-4} or more. Water which had been shaken with mud from Barnstable Harbor for some 3–4 hours also reversed the rheotaxis, but water in which 50 mud snails had stood for 3–4 hours in 150 cc had no effect in this experiment. The crab extracts elicited proboscis responses as described by Copeland (1918) and Carr (1967) as well as reversal of rheotaxis. Mud water reversed rheotaxis without eliciting the proboscis reactions at a frequency greater than was shown by controls. Results are given in Table IX.

Influence of physical factors Water containing a concentration of dissolved oxygen greater than that of the water in which the snails had previously been maintained, produced a significant tendency to move upstream, though the reversal of rheotaxis was less consistent than that produced by prey extracts or mud-waters. Batches of snails kept for one hour in seawater saturated with oxygen or nitrogen, and snails kept in aerated seawater were each tested in seawater previously saturated with oxygen, nitrogen or air. Currents were maintained with a nitrogen jet for the nitrogenated and aerated seawater, and with an oxygen jet for the oxygenated seawater. Results are given in Table X. All snails tended to move downstream, but snails transferred to conditions of greater oxygenation showed a significantly higher proportion of individuals moving upstream than did other snails.

TABLE IX

Effect of current bearing olfactory stimuli

Snails in circulating water

Additive	Concentration of extract	No. of snails	% moving upstream
Seawater control	—	20	5
Carcinus extract	5×10^{-5}	14	21
	2×10^{-4}	18	22
	7×10^{-4}	6	50
	1×10^{-2}	19	94
	3×10^{-2}	33	64
Seawater control	—	30	20
Libinia extract	1×10^{-5}	8	38
	1×10^{-4}	8	100
	1×10^{-3}	9	89
Seawater control	—	20	20
Mud water	4×10^{-4}	13	31
	4×10^{-3}	10	40
	1.2×10^{-2}	10	30
	2.3×10^{-2}	10	80
	4×10^{-2}	10	90

Similar results were obtained with snails on draining slopes.

Alteration of salinity to abnormally low or high levels reduces or abolishes the positive rheotaxis normally elicited by *Libinia* extract at concentrations of 1×10^{-4} and above. Normal laboratory tap seawater had a salinity of 31‰. Half strength

TABLE X

Effect of dissolved gases

	% of snails moving upstream in		
	Nitrogen saturated seawater	Aerated seawater	Oxygen saturated seawater
Snails from:			
Nitrogen saturated seawater	33	32	46
Aerated seawater	—	16	—
Oxygenated saturated seawater	11	32	20
Grouping the data		No. moving upstream	No. moving downstream
Snails moved into increased oxygen tension		28	59
Snails moved into same or reduced oxygen tension		14	75

χ^2 for 1 degree of freedom, applying Yates' correction = 5.6.
$0.05 > P > 0.01$.

seawater was prepared by dilution with distilled water. Hypersaline water was prepared by evaporation. Both hyposalinity (15‰) and hypersalinity (33.4‰) reduce or abolish positive rheotaxis, as shown in Table XI.

In 15‰ the behavior of snails is abnormal in that they take longer to extend the foot. During movement the anterior margin of the foot is frequently raised from the substrate and replaced. The whole foot is turned one way and the other to a greater extent than normally, and adheres less firmly to the glass. Scheltema (1965) found that the spontaneous activity of adult *Nassarius* was sharply reduced at salinities of 17‰ and below. In seawater of a salinity of 47‰ the animals emerged but did not stay upright. In seawater of 62‰ the animals remained firmly contracted inside their shells.

These experiments demonstrate the lability of the rheotactic response. Characteristics of the water indicating more favorable conditions, cause an upstream response and vice versa. The presence of food odors represents a more favorable

TABLE XI

Effect of salinity

	% moving upstream	
Salinity ⁰/₀₀	Clean seawater	Seawater with crab extract
15	26	37
28	—	80
31	17	57
33.4	—	20
38.7	—	5
47	Did not crawl	—
62	Remained in shells	—

source and normally causes an upstream movement which would lead to the location of the food. This response can be reversed if unfavorable physical factors are also present. To animals that have recently been fed, however, the presence of food odors does not represent a more favorable condition and they do not usually move upstream.

Since the threshold at which a reversal of rheotaxis occurs varies with the physiological state of the individual, when a population lies at the confluence of a number of streams, those streams with a strong stimulating capacity will attract most of the individuals in their path, while those with a weak odor will attract only individuals with a low threshold. Thus the population will appear to choose the stream with the strongest odor. This phenomenon can be employed to demonstrate the relative stimulating capacity of various effluents in experiments with Y tubes or multiple choice boxes.

Gregariousness

Because of the schooling and aggregation shown in the field it seemed desirable to see whether there was any chemical attraction between healthy mud snails. A four-way choice chamber consisting of four square bait boxes symmetrically disposed about a central square box into which snails were put at the start of an experiment was used. Water flowed into the bait boxes through flasks and out through a stand pipe in the center box. To test whether the attraction was dependent on the effluent produced only by well-fed animals, the water entering two of the bait boxes was lead through flasks containing equal numbers of fed and starved snails, respectively. The other two boxes served as controls being supplied with seawater at the same rate. To test whether the response was dependent on hunger, as was found with prey odors, fed and starved test animals were placed simultaneously in the center box, and were distinguished by painted marks on the shell. Four replicates in which each bait box served in turn for each effluent or control, constituted a balanced experiment, eliminating any inherent differences between the bait boxes. The results of two independent experiments (Table XII) showed that the snails were attracted by *Nassarius* effluent, particularly that from recently fed *Nassarius*. No difference in behavior was shown by fed and starved test animals.

TABLE XII

Gregarious effect

	Fed snails used as test		Starved snails used as test	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Percentage of snails entering bait box containing:				
Fed *Nassarius*	55%	49%	43%	46%
Starved *Nassarius*	14%	30%	23%	13%
Seawater control (mean of 2 boxes)	1%	0%	8%	3%
Left in starting box	29%	21%	18%	35%
Number of snails used	132	118	200	127

TABLE XIII

Effect of mud and feces

	Feces in left bait box	Feces in right bait box
Number entering bait box with mud and feces	2	3
Number entering control bait box	6	3
Number left in starting box	30	38

A further experiment was carried out to test whether the faeces of recently fed *Nassarius*, perhaps still carrying the odor of the food, were responsible for the attraction. This experiment was performed using the standard two choice (Y tube) apparatus. The results are given in Table XIII and show that mud and faeces left by fed snails held no attraction. A check was made of the gregarious response in the two way choice apparatus; 66 entered the box with an effluent from 100 well cleaned snails and only 5 entered the control box. On another occasion the effect was less marked, 119 entering the *Nassarius* bait box and 71 the control box, suggesting that individuals are not always attracted to each other. These experiments are apparently at variance with the result of an earlier experiment described in the section on rheotactic behavior; in which no reversal of rheotaxis was elicited

TABLE XIV

Upstream movement in response to water stood over deposits of different origin

Source of deposit	No. of animals used	% moving upstream
Barnstable Harbor 24. VII. 67		
Nassarius bank		
Surface	10	80*
Sub-surface	10	80*
Submerged	9	77*
Diatom bank	30	66*
Purple bacteria bank	19	26
Laboratory seawater control	48	29
Barnstable Harbor 2. VIII. 67		
Nassarius bank	10	100*
Purple bacteria bank	10	90*
Diatom bank	10	70*
Control Seawater	30	13
Sandwich Marshes		
Surface Mud (brown)	10	80*
Sub-surface Mud (black)	18	78*
Laboratory seawater control	61	34

Key: "Nassarius bank": A small bank of grey silty sand on which *Nassarius obsoletus* was found in quantity between 14.7. and 2.8.67, and after 17.8.67.
"Diatom bank": A bank of firm sand just below the Spartina grass with abundant *Gemma* and *Hydrobia* and rich flora of diatoms, green and blue green algae.
"Purple bacteria bank": Edges of a channel adjacent to the Spartina marsh with diatoms and abundant purple bacteria.
* Significant difference from control.

TABLE XV

Effluent choice experiments to seawater passed through various deposits

Experiment	Number of animals used	Source of deposit	Percentage moving into effluent from deposit
1	270	Nassarius bank	30%
		Diatom bank	25%
		Purple bacteria bank	44%
		Laboratory seawater control	1%
2	157	Purple bacteria bank	74%
		Laboratory seawater control (3 replicates)	9%

by water in which mud snails had been stored. It is possible that in the choice experiments the animal is presented with a fluctuating olfactory stimulus to which it is more sensitive. Alternatively, the substance which the animals emit may be labile or the response may not always be given.

Influence of substances derived from deposits *Nassarius* shows a reversal of rheotaxis when exposed to water that has been in contact with sandy-mud (see section on rheotactic behavior). However, the response was not reliably given unless the snails had previously been starved in the laboratory. Seawater was stored over bottom deposits for 24 hours or shaken up with them for 3–4 hours and tested for its capacity to reverse the rheotaxis of starved laboratory animals in fingerbowls with a circulation maintained by an air jet (method 1 of previous experiments on rheotaxis). The results given in Table XIV show that deposits from the surface, from the sub-surface, from aerobic or from anaerobic conditions, and deposits widely differing in their microflora, all contain substances that stimulate the snails to move upstream. The only exception was a single sample of a deposit containing large quantities of purple bacteria. It is possible that some incidental physical property of this sample caused it to be unattractive so that the mud factor was masked.

Similar results were obtained by passing seawater through deposits, each in a conical flask, and thence into "bait boxes" of a 2 or 4 way choice apparatus. In two such experiments (Table XV) the snails showed a clear preference for water that had passed through each of three different deposits over a laboratory seawater control; one deposit was from the bank where the mud snails were commonly

TABLE XVI

Inactivation of seawater containing mud factor by charcoal filtration

Treatment of water	Number of snails used in test	% moving upstream
Stood over Barnstable Harbor mud 2 days and filtered	27	89
Stood over mud 2 days and filtered through activated charcoal	10	20
Laboratory seawater control	26	19

TABLE XVII

Response of Nassarius *to effluent from natural and pre-boiled mud*

Effluent from	Percentage in prey box	
	Experiment 1 (55 animals)	Experiment 2 (46 animals)
Natural mud	70%	89%
Boiled mud	4%	7%
Laboratory seawater control (2 replicates)	13%	2%

found, and the other two from areas from which they were absent. One of these possessed a high content of purple bacteria, and the other of diatoms, *Enteromorpha* and blue green algae.

There was no evidence from either experiment that the deposits on which *Nassarius* congregated yielded the most effective effluents. The mud from the Sandwich area, where *Littorina littorea* occurs in quantity and there are no *Nassarius,* was as effective in reversing the rheotaxis as Barnstable deposits where *Nassarius* is common.

The effective agent in seawater passed over a muddy deposit can be inactivated by filtering the water through charcoal to which it is presumably absorbed (Table XVI), or by boiling the mud beforehand (Table XVII).

Substrate choice

Though a rheotactic response was given to seawater that had been in contact with mud, no reaction to mud factors specific to the areas occupied by *Nassarius* could be demonstrated. Mud snails were therefore offered a choice of various

TABLE XVIII

Substrate selection

Deposits tested	% of *Nassarius* present in:—		
	Exp. 1 whole deposits	Exp. 2 coarse fraction	Exp. 3 sieved fine fraction
Nassarius bank			
Surface intertidal	38*		
Sub-surface intertidal	2	8	4
Surface submerged	22*		
Diatom bank			
Surface	31*	76*	38*
Purple bacteria bank			
Surface	7**	10†	58**
Number of snails in choice experiment	165	50	25

** Rich in purple bacteria.
* Rich in diatom or algal material.
† Rich in detritus material.
The remaining deposits were predominantly inorganic.
For details of deposits see Table XIV.

sediments to see whether they would accumulate on some more than on others. Some 25 or more individuals were placed at the center of a tray containing a number of samples of different deposits each filling a Petrie dish; the dishes and experiments were replicated to ensure adequate balance for positional differences. The experiments were performed mostly in the dark but identical results were obtained in some experiments in the light. The results of three groups of experiments on the three main types of deposit, and on fractions obtained by sieving are given in Table XVIII. Microscopical examination revealed that certain of the surface deposits were rich in diatoms and algae and others in purple bacteria. The subsurface deposits, as expected, were mainly inorganic. As can be seen from the table, the snails accumulated mainly on the diatomaceous deposits, where they were observed feeding, and on the fine fraction of the bacteria-rich deposit, but they avoided the subsurface deposits. Moreover as the surface flora of the deposits was removed by the snails' activity they tended to migrate from the diatom rich sand, where the algae were mainly at the surface, to the muds which were rich throughout in bacteria. As *Nassarius obsoletus* is known to be a facultative scavenger and deposit feeder, the conclusion appears to be that in the laboratory the snails congregate on deposits with the greatest abundance of food rather than on a particular deposit type. Wieser (1956) obtained similar results for the cumacean *Cumella vulgaris*.

DISCUSSION

Before schooling can occur, animals must assemble in a certain locality. The laboratory studies suggest three possible olfactory stimuli which, by causing animals to move upstream, might bring them together at the source of the stimulus.

First, the influence of the attractive factor which is continuously eluted when seawater flows over mud would cause snails to move upstream as far as the point at which a threshold concentration exists, or until opposed by some unfavorable influence. Thus in the sub-threshold concentrations of the mud factor, which might exist near the source of runnels draining off the marsh, snails would move downstream, whereas further down the drainage channels where a higher concentration of mud factor built up, they would move upstream. Downstream movement in the upper reaches of the harbor cannot be attributed to lowered salinity, since the hydrographic study of Ayers (1959) showed that in the area where *Nassarius* is found the salinity departs very little from 31% at any state of the tide.

Secondly, attraction to a localized source of olfactant, for example the effluent from damaged animal tissues stranded on the mud banks, would also lead to an accumulation of snails. This kind of accumulation can be seen on intertidal flats, but the character of such aggregations is apparently different from those described by Jenner (1956, 1957, 1958, 1959). Nor are the majority of schools observed on the flats associated in any way with visible localized sources of olfactant.

Finally, the attraction of individuals by groups of living, undamaged mud snails would be most effective in recruiting snails once a group had been established.

A further reaction likely to lead to congregations of snails is the observed reversal of geotaxis on submergence. By moving down from the damp surfaces of the banks and moving up from below the water level, animals would accumulate at the water's edge in the manner seen in the field.

The orientation of schools was seen to be in the direction which would be predicted from laboratory experiments. For example, schools moved downhill and accumulated in damp hollows on the banks. On reaching the water's edge they moved along it, usually against the current.

In the laboratory much variation of individual behavior towards the same stimulus was observed, whereas the members of a school were seen to move together. In the middle of a school conformity could be purely mechanical, opposition to the direction of movement being physically impossible without disrupting all movement of the school. At the edges, the conforming tendency demonstrated in the laboratory may be important. Any stimulus which induces entering snails to conform with the direction of movement of a school must be polarized in that direction; chemical stimuli are therefore unlikely to be involved. A visual response to the images of other snails at a distance is ruled out by the lack of an optomotor response, although a light-compass reaction to the passing shadows of nearby snails might perhaps occur. The tactile stimulus of contact of the soft parts of the animal, including foot and siphon, is probably of greatest importance in inducing conformity.

Scheltema (1961) showed that the veligers of *Nassarius* responded to seawater which had stood over favorable muds by dropping to the bottom and metamorphosing. The active agent was water-soluble, heat-labile, and unaffected by ordinary filtration. The mud extracts to which adults respond also have these properties. The effectiveness of mud extracts in reversing the rheotaxis of adults is destroyed by filtration through activated charcoal, rendering it likely that the active agent is chemical.

The responses to mudwaters of both larvae and adults may have the function of habitat selection. The adult response to mudwaters was less drastically reduced when they had recently fed than was the response to effluents from damaged animal tissues. Moreover when placed in tissue effluents the snails frequently exhibited proboscis reactions whilst moving upstream. When placed in mudwater the proboscis was extruded with no greater frequency than in clean seawater, suggesting that the positive rheotaxis induced by mudwater may not be primarily a food-seeking response.

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SUMMARY

The reactions of specimens of *Nassarius obsoletus* exposed individually to controlled laboratory stimuli are as follows:

1. *Nassarius* shows a light-compass reaction, but no optomotor response.

2. On damp slopes *Nassarius* moves downhill. Submerged *Nassarius* is generally geonegative but its response is altered by a nearby light source.

3. In clean seawater *Nassarius* moves downstream.

4. Addition of effluents from damaged animal tissues or from mud causes a reversal of rheotaxis.

5. A small but significant upstreaming response is elicited by water of raised oxygen concentration.

6. The upstreaming response to olfactory stimuli can be abolished when unfavorable stimuli (hypo- or hypersalinity) are simultaneously present.

7. Water which has passed over living, intact *Nassarius* is attractive to other individuals of the species *Nassarius*, the attraction not being due to faeces.

8. When simultaneously presented with substrates of different kinds, mud snails accumulate on those richest in diatoms and bacteria.

9. The behavior of an individual *Nassarius* is greatly affected by the behavior of others around it. Members of a group of *Nassarius* in the laboratory or in the field show a strong tendency to conform in their direction of movement, thus producing schooling. The orientation of schools in the field was that expected from the behavior of the majority of individuals in the laboratory.

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FEEDING ACTIVITY IN *ECHINASTER* AND ITS INDUCTION WITH DISSOLVED NUTRIENTS[1]

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While many species of starfishes are notorious for their predaceous attacks on edible shellfish, others, particularly those belonging to the family Echinasteridae, have been considered economically harmless. Indeed, until fairly recently we remained almost completely ignorant of the normal food habits and nutritional mechanisms of this latter group of animals. In 1960, Anderson published the first truly accurate and detailed account of the histology and structure of the digestive system of a member of this family, *Henricia leviuscula.* He also presented a reasonable analysis of the functioning of this system in the collection and processing of nutritive materials. Particularly, he called attention to the fact that the Tiedemann's pouch, a conspicuously developed outgrowth of the pyloric stomach found beneath each digestive gland, acts as "a hydrodynamic organ or flagellary pump of prodigious effectiveness" (p. 393). These structures transport material from the flagellary tracts of the stomach and rapidly distribute it to secretory and absorptive areas in the digestive glands, maintaining it in continuous circulation through these organs "until digested, absorbed, or eliminated" (p. 392). To support the hypothesis that *Henricia* is primarily a particulate feeder, Anderson exposed two specimens of *Henricia sanguinolenta* to suspensions of dyed *Mytilus* sperm. These were seen to be drawn up into the partially opened mouth of the starfish, collected as streams in the radial peristomial grooves, or entangled in strands of mucus that also moved into the mouth. The dyed food particles were subsequently found at various places in the Tiedemann's pouches.

While Anderson pointed out that, in feeding, the cardiac stomach of *Henricia* probably could not be everted to the extent of the stomachs of such starfishes as *Asterias* and *Patiria,* he did note that in some specimens stomach vesicles press through the mouth as lobe-like structures extending about a millimeter. The extension of the stomach as a button-like protuberance has been seen by a number of other investigators, including the author. Vasserot (1961) notes this ability in both *Henricia sanguinolenta* and *Echinaster sepositus.* In fact, he concludes that these two species are primarily macrophagous feeders restricted almost exclusively to a diet of sponges, or are at least stimulated to assume a feeding posture by sponge fragments.

In further studies on *Henricia sanguinolenta,* Rasmussen (1965) has confirmed Anderson's opinion that this species is primarily a filter feeder, capable of removing diatoms and flagellates from suspensions at high rates. He concludes that Vasserot misinterpreted his observation in that *Henricia,* even when mounted on a sponge, was not significantly harming it, but rather benefiting from the sponge's water currents while functioning as a suspension feeder. He refers to this relationship as a state of "energy commensalism" (p. 187).

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[1] Supported by NSF grant GB 6906.

Other evidence is available that neither particle-suspension nor macrophagous feeding fully explain the nutrition of these starfishes. Stephens and Schinske (1961) first noted that *Henricia sanguinolenta* (along with many other invertebrates) could take up dissolved glycine from sea water. Ferguson (1963, 1967a, 1967b, 1968b) has since explored in much greater detail uptake from dilute solutions of free amino acids and sugars by starfish. Ferguson has emphasized that these substances may significantly contribute to the maintenance of the superficial tissues of the body, which otherwise might have difficulty obtaining sufficient nutrients from internal sources.

While in Ferguson's experiments, free dissolved nutrients were generally not taken into the digestive system and deeper regions of the body, several instances of such uptake were noted in both *Henricia sanguinolenta* and *Echinaster echinophorus*. These instances occurred sporadically when specimens of these species were exposed to fairly high concentrations of glucose. They indicate the possibility that completely dissolved nutrients, when present in sufficient concentration, can stimulate the filter pumping machinery of the organisms into operation. The observation of such a response is most interesting in that it could be interpreted as showing that filter-pumping by starfish is a significant function in a variety of nutritional roles, including the uptake not only of suspended material, but also of dissolved substances from different sources. These substances could be obtained from external digestion, decaying (or autolyzing) carrion and detritus, or simply organically rich sea water. The present study was carried out in order to provide further information on the feeding behavior of *Echinaster echinophorus* and to more fully delineate the ability of dissolved nutrients to stimulate filter-pumping in this species.

Materials and Methods

In addition to collecting observational data on the normal feeding habits of the Echinasteridae, experimental evidence was sought to more clearly define the nutritional capabilities of these starfish. The primary approach used in these experiments was to expose animals to various concentrations of different dissolved nutrients to permit determination of the adequate stimuli for inducing them to engage in the filter-pumping "feeding" process. As neither the method of Anderson (1960), involving direct observation of the ingestion of particles, nor that of Rasmussen (1965) involving colorimetric observation on food organism cultures exposed to the starfish, appeared to be suitable for determining whether the experimental animals had engaged in filter-pumping during an experimental period, another procedure had to be devised. The method finally chosen was a simplified, essentially qualitative version of that previously used in nutrient utilization studies (Ferguson, 1967a).

Starfish, *Echinaster echinophorus,* were collected from tidal grass flats and kept on a sea table in recirculating water for several days. (Specimens were examined by Maureen Downey of the U. S. National Museum and tentatively assigned by her to this species, pending revision of the group. In previous papers by the author the same form has been referred to as *Echinaster spinulosus.*) Only moderate size individuals were selected for further study. As the experiments were all performed during the midsummer period, the gonads of these specimens were in a relatively early stage of redevelopment from the spring spawning, and the digestive glands occupied most of the coelomic cavity. Pairs of specimens were placed together in finger bowls containing 100 ml of filtered sea water from the same sea table, to which had been added a carefully measured quantity of the nutritive substance under investigation, and a small quantity of high specific activity C^{14}-labeled nutrient. No attempt was made to accurately measure the latter, as it contributed negligible mass, and the variety of labeled substances used were obtained from several sources of different strengths. The only concern was that sufficient labeled marker was present to be easily detected in the samples if the nutrients were taken up. The initial high purity of both the labeled and unlabeled compounds was verified from data provided by the manufacturers, and all the compounds were stored under optimum conditions to maintain stability.

Each experiment was allowed to continue for 4 hours. Following this period the animals were removed from the bowls, rinsed in 3 changes of sea water, and dissected into two parts—the digestive glands (including the Tiedemann's pouches) and the remaining structures (chiefly body wall). Each of these was then placed in a test tube together with 1 ml of 10 per cent NaOH. After dissolving the tissues by heating the test tubes in boiling water for a few minutes, the resulting digests were decanted into 1-inch steel planchets, dried overnight in an oven, and counted as "infinitely thick" samples in an automatic G-M counter. The data were evaluated subjectively, primarily by comparing the level of radioactivity found in the digestive glands with that found in the body wall of each specimen.

A list of the nutrients tested may be found in Tables I and III. Each of the compounds listed in Table I was used in conjunction with its uniformly C^{14}-labeled counterpart. In Table III, lactalbumin hydrolysate was used as a representative mixture of amino acids. Its uptake was tested with a commercial mixture of uniformly labeled C^{14}- amino acids (representing a synthetic algal protein hydrolysate) obtained from New England Nuclear Corp., Boston, Mass. As examples of proteins, gelatin, casein, and bovine serum albumin were tested with a purified algal protein-C^{14}, also obtained from New England Nuclear Corp.

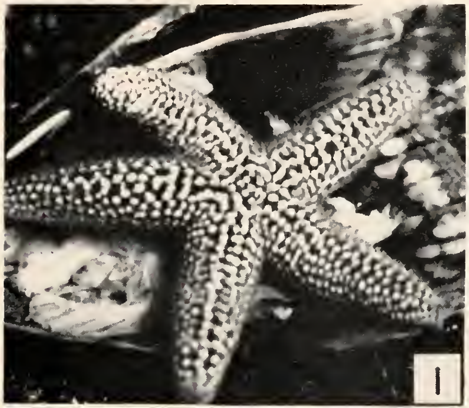
OBSERVATIONS

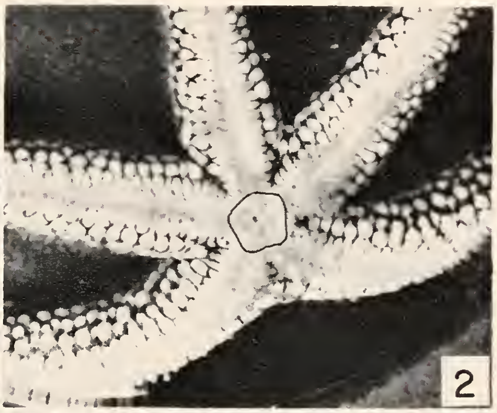
General observations on feeding

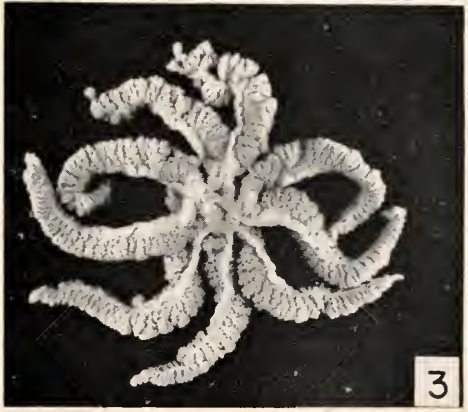
As was noted by Vasserot (1961), *Echinaster* is often found in the field in association with sponges. In the present collections of 1136 specimens, 369 (32.5%) were found in such associations, and with their stomachs partially everted. In some cases the sponges showed signs of damage, but generally they were still quite healthy. The relationship with sponges does not appear to be exclusive, however, as in the same collections 44 specimens (3.7%) were found in a feeding position on detritus-rich sand, 23 (2.0%) on algae, 21 (1.9%) on ascidians, 16 (1.4%) on snails, and 1 on an annelid. The remaining 662 (58.3% showed no evidence of feeding activity, and often appeared to be in transit across the substrate. In captivity, specimens will readily settle in a feeding position on shellfish which have been opened for them (Fig. 1). They will also frequently attack more defenseless species, such as sunray clams (*Macrocallista*) and sand dollars

(*Mellita*). While victims of such attacks often show signs of being damaged by digestive activity, they are practically never completely devoured. Rather, the remains are abandoned after about 24 hours.

In practically all cases when a captive specimen is seen in a feeding posture, its stomach is found to be everted as a distinctive button-like structure. Such eversion was also noted in many of the experiments with soluble nutrients (Fig. 2). Almost invariably, when eversion was seen in the experiments, significant radioactivity was later located in the digestive glands. In many cases, however, radioactivity was found in the digestive glands of specimens which were not observed with everted stomachs. It is quite possible that in these cases brief periods of eversion may have been overlooked.







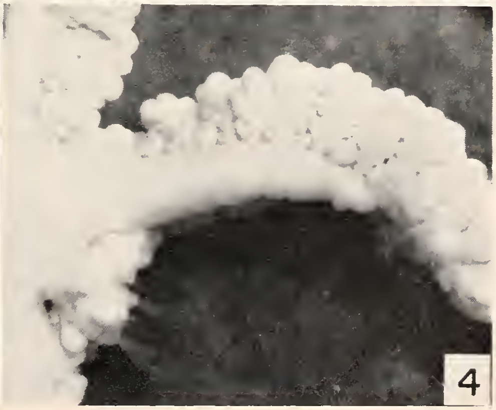


FIGURE 1. *Echinaster* is a typical feeding position on an opened pen shell clam (*Atrina*).

FIGURE 2. Oral view of *Echinaster* with everted stomach (outlined). Eversion was induced by placing the animal in 100 mg % lactalbumin hydrolysate.

FIGURE 3. Oral view of dissected digestive system of *Echinaster*. Mouth and small pyloric stomach may be seen in center. Note that the Tiedemann's pouch extends for $\frac{1}{3}$ to $\frac{1}{2}$ the length of each digestive gland.

FIGURE 4. Detail of Tiedemann's pouch showing its attachment to the digestive gland and the pyloric stomach.

Anatomy and histology

Dissection and routine histological study reveal that the digestive system of *Echinaster* is very similar to that described for *Henricia* by Anderson (1960). Each digestive gland is attached to the pyloric stomach by a well-developed Tiedemann's pouch (Figs. 3 and 4). The pouch is divided into a series of diagonal channels by "seam-cell" adhesion-bands. These channels are very heavily lined with cilia (flagella), and doubtless do form a very powerful pumping organ. As in *Henricia,* the epidermal nerve plexus and associated "spindle nerve cells" are very well developed in the floor of the Tiedemann's pouch, along the adhesion-bands, and along the line of attachment of the Tiedemann pouch and the digestive gland.

TABLE I

Relative uptake of dissolved labeled nutrients by body wall and digestive glands

Nutrient	CPM $\left(\frac{\text{Digestive glands}}{\text{Body wall}}\right)$											
	Concentration 1.0 m*M*				2.5 m*M*				5.0 m*M*			
Glycine	$\frac{34}{966}$	$\frac{350}{991}$	$\frac{17}{411}$	$\frac{143}{431}$	$\frac{73}{1375}$	$\frac{102}{1352}$	$\frac{17}{1543}$	$\frac{616}{2120}$	$\frac{500}{3320}$	$\frac{948}{3320}$	$\frac{315}{262}$	$\frac{225}{259}$
L-alanine	$\frac{7}{285}$	$\frac{11}{393}$	$\frac{14}{195}$	$\frac{11}{218}$	$\frac{60}{93}$	$\frac{36}{74}$	$\frac{43}{863}$	$\frac{28}{721}$	$\frac{410}{1984}$	$\frac{1069}{1415}$	$\frac{9}{82}$	$\frac{52}{88}$
L-serine					$\frac{42}{776}$	$\frac{85}{638}$			$\frac{81}{1149}$	$\frac{184}{1177}$		
L-valine	$\frac{22}{683}$	$\frac{27}{729}$	$\frac{77}{734}$	$\frac{42}{580}$	$\frac{16}{668}$	$\frac{37}{500}$	$\frac{42}{442}$	$\frac{46}{488}$	$\frac{509}{338}$	$\frac{20}{363}$	$\frac{61}{304}$	$\frac{68}{388}$
L-leucine	$\frac{93}{961}$	$\frac{866}{1233}$	$\frac{42}{974}$	$\frac{11}{1039}$	$\frac{141}{642}$	$\frac{348}{700}$	$\frac{1074}{311}$	$\frac{251}{350}$	$\frac{334}{848}$	$\frac{140}{581}$	$\frac{295}{499}$	$\frac{1889}{514}$
L-isoleucine	$\frac{10}{145}$	$\frac{31}{200}$	$\frac{25}{391}$	$\frac{90}{586}$	$\frac{43}{160}$	$\frac{8}{180}$	$\frac{72}{511}$	$\frac{65}{346}$	$\frac{219}{150}$	$\frac{123}{99}$	$\frac{302}{288}$	$\frac{42}{340}$
L-phenylalanine	$\frac{505}{661}$	$\frac{217}{798}$	$\frac{445}{179}$	$\frac{391}{161}$	$\frac{988}{533}$	$\frac{53}{364}$	$\frac{312}{109}$	$\frac{1227}{83}$	$\frac{1258}{407}$	$\frac{1062}{372}$	$\frac{294}{58}$	$\frac{273}{56}$
L-arginine	$\frac{8}{298}$	$\frac{10}{225}$	$\frac{4}{177}$	$\frac{7}{216}$	$\frac{8}{186}$	$\frac{9}{198}$	$\frac{17}{254}$	$\frac{11}{187}$	$\frac{10}{188}$	$\frac{7}{125}$	$\frac{9}{165}$	$\frac{8}{146}$
L-lysine					$\frac{48}{358}$	$\frac{10}{236}$	$\frac{49}{522}$	$\frac{120}{499}$	$\frac{23}{491}$	$\frac{16}{399}$	$\frac{66}{415}$	$\frac{9}{383}$
L-glutamic acid	$\frac{10}{235}$	$\frac{6}{145}$	$\frac{6}{114}$	$\frac{5}{90}$	$\frac{257}{1148}$	$\frac{89}{1229}$	$\frac{14}{165}$	$\frac{13}{147}$				
D-glucose	$\frac{15}{282}$	$\frac{427}{277}$	$\frac{55}{249}$	$\frac{33}{161}$	$\frac{3120}{286}$	$\frac{7057}{748}$	$\frac{2223}{248}$	$\frac{561}{247}$	$\frac{204}{892}$	$\frac{2185}{250}$	$\frac{1990}{327}$	$\frac{1426}{329}$

These nervous elements may be very significant in controlling the pumping activity of the pouch.

Pumping and the uptake of soluble nutrients

When the starfish were exposed to various concentrations of soluble nutrients, very distinctive patterns of response were noted (Tables I, II, III). In essentially all cases the body wall (epidermis) took up observable quantities of the nutrients. Such a result was expected from previous experiments (Ferguson, 1963, 1967a, 1967b, 1968b), although this represents the first time that the epidermal uptake of many of the specific compounds has been confirmed. Varying degrees of radioactivity were also found in the digestive glands. Some of this was doubtless due to unavoidable contamination during the dissection of the organs from the animal; most of the remainder probably reached the organs in intervals of ciliary pumping by the Tiedemann's pouches during the 4-hour exposure period. In extreme cases, where ciliary pumping apparently persisted over most of the exposure period resulting in a large percentage uptake by the digestive glands, a markedly lower incorporation could be noted in the body wall, doubtless because less activity remained in the media to be taken up by these elements. Likewise, when very high concentrations of nutrients were present, reduced uptake of tracer was observed in all parts—probably due to the saturation of the absorptive mechanisms. This phenomenon is most noticeable in Table III with the higher concentrations of lactalbumin hydrolysate.

TABLE II

Feeding interpretation of experiments listed in Table I

	Concentration 1.0 m*M*	2.5 m*M*	5.0 m*M*
Glycine	− (+) − (+)	− − − (+)	− (+) + +
L-alanine	− − − −	+ + − −	(+) + − +
L-serine		− −	− −
L-valine	− − − −	− − − −	+ − (+) −
L-leucine	− + − −	(+) + + +	+ (+) + +
L-isoleucine	− − − −	(+) − − −	+ + + −
L-phenylalanine	+ − (+) −	+ − + +	+ + + +
L-arginine	− − − −	− − − −	− − − −
L-lysine		− − − −	− − − (+)
L-glutamic acid	− − − −	(+) − − −	
D-glucose	− + (+)(+)	+ + + +	+ + + +

Each mark represents the behavior of a separate specimen: − = insignificant amount of feeding activity; (+) = possibly significant amount of feeding activity; + = probably significant amount of feeding activity.

In light of the aforementioned variables—possible contamination of tissue samples, individual variations in the duration of the pumping, and competitive inhibition of uptake—arbitrary criteria had to be established to simplify the task of interpreting

TABLE III

Relative uptake of dissolved labeled nutrients by body wall and digestive glands and interpretation

Nutrient	Concentration mg%	CPM $\left(\frac{\text{Digestive glands}}{\text{Body wall}}\right)$				Feeding interpretation*
Amino acid-C^{14} mixutre & Lactalbumin hydrolysate	0	$\frac{288}{9788}$	$\frac{197}{11846}$	$\frac{40}{3832}$	$\frac{69}{3583}$	− − − −
	10	$\frac{84}{4807}$	$\frac{130}{4348}$	$\frac{26}{1022}$	$\frac{833}{1022}$	− − − +
	25	$\frac{347}{608}$	$\frac{2378}{567}$			+ +
	50	$\frac{596}{627}$	$\frac{4818}{701}$			+ +
	75	$\frac{680}{635}$	$\frac{3016}{566}$			+ +
	100	$\frac{92}{1472}$	$\frac{1302}{1135}$	$\frac{2765}{588}$	$\frac{374}{425}$	− + + +
	125	$\frac{1986}{474}$	$\frac{1302}{401}$			+ +
	150	$\frac{921}{327}$	$\frac{1302}{401}$			+ +
	500	$\frac{51}{223}$	$\frac{58}{205}$			(+)(+)
	1000	$\frac{69}{179}$	$\frac{105}{240}$			(+)(+)
Algal Protein-C^{14} & Gelatin	10	$\frac{30}{142}$	$\frac{34}{314}$	$\frac{24}{173}$	$\frac{20}{179}$	(+) − − −
	50	$\frac{63}{156}$	$\frac{28}{146}$	$\frac{63}{105}$	$\frac{24}{150}$	+ − + −
Algal Protein-C^{14} & Casein	10	$\frac{28}{412}$	$\frac{40}{101}$	$\frac{22}{93}$	$\frac{864}{120}$	− (+) − +
	50	$\frac{26}{48}$	$\frac{41}{59}$	$\frac{60}{130}$	$\frac{38}{70}$	+ + + +
Algal Protein-C^{14} & Serum Albumin	10	$\frac{30}{294}$	$\frac{20}{258}$	$\frac{23}{289}$	$\frac{18}{219}$	− − − −
	50	$\frac{29}{277}$	$\frac{14}{275}$	$\frac{15}{180}$	$\frac{16}{232}$	− − − −
Algal Protein-C^{14} alone	(Negligible)	$\frac{43}{311}$	$\frac{45}{255}$	$\frac{23}{213}$	$\frac{554}{513}$	− − − +

* See Table II for clarification of symbols.

the experimental results. Therefore, when the level of radioactivity found in the digestive glands was over 40% of that found in the body wall, a significant degree of pumping (feeding) was considered to have probably occurred. Less than 20% was considered insufficient evidence for feeding, while 20–40% was considered as possibly significant feeding activity. These interpretations are presented in Tables II and III. On this basis, very marked differences in the stimulating effects of different substances may be noted.

Discussion

There can be little doubt that these starfish are capable of making effective use of a great variety of soluble materials, absorbing them not only into their exposed epidermal tissues, but also, actively pumping them up and assimilating them into their internal digestive organs. A measure of the efficiency of the latter process can be seen in the observation (Table I) that some of the glucose-exposed specimens took up as much as 10 times the dissolved nutrient internally as they did into the exposed superficial tissues.

In most cases, however, much smaller ratios of uptake between the two areas are apparent. Some "background" activity is, of course, always found in the digestive glands. This usually amounted to several per cent of the activity found in the body wall and (as previously stated) was probably due to unavoidable contamination of the samples and other causes. But beyond this low level, considerable variation exists in the uptake of nutrients into the digestive glands even of specimens exposed to the same conditions (Tables II and III). Thus, it seems likely that the individual specimens have considerable control over their activities. They do not pump up material continually, but take it up over periods of varying lengths of time. The ingestion appears to be a relatively high order response on the part of the animals, and not a simple reflex activity entrained to a minimal adequate stimulus.

The ability of starfish to limit ciliary pumping to periods when it might be most productive in obtaining nutrients would, of course, be a significant advantage to them in terms of conservation of energy. It is quite clear from Tables II and III that higher concentrations of dissolved nutrients are generally much more successful than lower ones in stimulating pumping. Thus, concentrations of 25 mg% or greater of mixed amino acids (Table III) almost invariably induce significant uptake, while only one animal in four responded positively to a concentration of 10 mg%. When one considers the energy that would be required to pump up the water through the narrow digestive channels and extract the dissolved nutrients from it, it is doubtful that concentrations of nutrients much below this order of magnitude could be utilized at a net energy gain. Very much lower concentrations of soluble nutrients are taken up by the superficial tissues, however, where the animal could rely almost entirely on the natural circulation of the environmental fluids and would need expend energy only for the active transport process (Ferguson, 1967a, 1967b).

Not all the compounds tested were equal in their ability to stimulate pumping. Glucose apparently is a most efficient stimulus, being very effective even at a concentration of 1.0 m*M* (Table II). It is not surprising, then, that it was with this compound that the pumping reaction was first evoked in *Henricia* (Ferguson, 1967a). Most of the neutral amino acids appear to be moderate stimulators, although a good deal of variation exists between them. Leucine and phenylalanine were particularly efficient, while no positive response at all was obtained in the four experiments with serine. The other neutral amino acids gave rather mixed results. The basic amino acids, arginine and lysine, and the acidic glutamic acid produced very little evidence of pumping activity.

Special note should be made of the results obtained with glutamic acid. Although it is relatively limited in its solubility, this amino acid has proven to have a number of interesting and as yet unexplained properties in echinoderm physiology. In previous experiments with *Echinaster,* for example, it appeared to enhance the serosal transport of other amino acids into the digestive glands, in spite of its own modest rate of transport (Ferguson, 1968a). It also produced both stimulatory and inhibitory effects on the muscle tone of these organs (Ferguson, 1966). In extracts of digestive glands and ovaries it is one of the few amino acids consistently present (Ferguson, unpublished). In *Asterina* it has been implicated as an inhibitor of a spawning substance obtained from the radial nerves (Ikegami, Tamura, and Kanatani, 1967). In light of these findings, one might expect glutamic acid to play at least some role in controlling the nutritional activities of these species. Thus, even the present essentially negative findings may prove to have significance as more information is obtained.

While many simple, low-molecular weight substances are apparently sought and taken up by starfishes, there is great uncertainty about the role of large molecules, particularly proteins, in this regard. One would, of course, expect predaceous species, such as *Asterias* and *Pisaster,* to take up these substances in conjunction with their well-known feeding habit of digesting bivalves in their shells. The only modern account of direct evidence for the uptake of soluble protein by a starfish is provided by Araki (1964), who notes that the everted stomach of *Patiria* will take up albumin. While this species possesses a Tiedemann's pouch system, it apparently is much less efficient than that found in the Echinasteridae (Anderson, 1960). This starfish feeds by extruding its enormous stomach over the sea bottom and devouring whatever is covered.

If *Echinaster* can respond so effectively to low-molecular weight amino acids and sugars, it would seem likely that it would also respond positively to dissolved proteins. The experiments which were carried out to verify this fact, however, were not fully successful.

The algal protein-C^{14} used as a label in these experiments was a highly purified product, but was only sparingly soluble in sea water. After its use, significant levels of radioactivity were found in the body wall (and in some cases the digestive glands) of animals exposed to it, but this activity could have been picked up in other ways. For example, it may represent protein simply adsorbed on the body surface, or the uptake of amino acid obtained from the breakdown of the labeled protein. Such breakdown could be due to the action of the well-developed secretory glands found in the epidermis, although no enzymatic activity has yet been observed in the products of these glands. Enzymes could also be released into the medium via the mouth. Bacterial decomposition of the protein during the four-hour experimental period is also a possibility.

In spite of these problems, some tentative conclusions can still be reached from the data obtained (Table III). None of the proteins tested, for example, appeared to be as effective in stimulating feeding as did the equivalent amount of free amino acid. Most of the positive reactions recorded were in reality minimal effects. Only in two of the twenty-eight experiments did greater amounts of radioactivity become localized in the digestive organs than in the body wall. And even in one of these two cases the positive results were obtained without the benefit of additional proteins added to the algal-C^{14} solution. These two cases, however, do indicate fairly clearly that at least in some circumstances free proteins can be removed from sea water and utilized by this species.

In summary, the current study demonstrates that *Echinaster,* like *Henricia,* possesses an efficient pumping mechanism. These animals can be stimulated to turn on this pump by the presence of free amino acids, glucose, and possibly protein in their surrounding medium, and can efficiently take up and absorb soluble compounds into their internal digestive organs. While this process is an intermittent one, generally requiring certain minimal concentrations of the dissolved nutrients, these same nutrients may be continually absorbed by the superficial tissues, even from extremely dilute solutions.

Furthermore, behavioral characteristics of the species serve to greatly enhance the possibility of obtaining such soluble nutrients. While the often-seen association of *Echinaster* with sponges might indeed represent "energy commensalism," it may well also represent a form of parasitism. The everted stomach may release enzymes which would act to produce low molecular products in a very localized region where they would be pumped up by the starfish. Loosely organized sponge tissues do not very readily show this damage, but such an effect is clearly evident as whitish splotches on sand dollars attached by this species in captivity. Such external enzymatic activity need only be of secondary importance, however, as the starfish can be equally efficient in picking up naturally released products and particulate materials. These might emanate from the bodies of sponges or from other soft-bodied invertebrates which may be attacked, or be obtained from carrion, detritus, or even organic-rich sea water.

The author acknowledges the able assistance of Mr. William Tench in preparing many of the samples used in this study. Mr. Lyman Goodnight and Miss Karen Brady assisted in the fieldwork.

SUMMARY

1. *Echinaster echinophorus* possesses a digestive system very similar to that of *Henricia.* Its Tiedemann's pouches are well developed and appear to function as pump organs.

2. About one third of the specimens seen in the field are found in a feeding posture on sponges (even though the sponges do not appear to be greatly harmed). Occasionally specimens are also found with everted stomachs on sand, algae, ascidians, molluscs, and other organisms. Captive specimens will assume a feeding position on opened clams and other defenseless invertebrates, but rarely completely devour them.

3. Tracer experiments demonstrate that various dissolved amino acids, glucose, and possibly proteins are taken up continually by the exposed superficial tissues of

the body. If present in suitable concentration (usually about 25 mg%) most of these substances will also be taken up into the internal digestive organs, presumably because they stimulate the animals to engage in filter-pumping.

4. Of the substances that have been studied, glucose is the most effective stimulator of the pumping process, followed by the neutral amino acids (particularly leucine and phenylalanine). The charged amino acids—arginine, lysine, and glutamic acid—are very poor stimulators, as are apparently several types of protein.

5. It is concluded that the digestive apparatus of *Echinaster* can function effectively in collecting nutrients from a variety of sources. It can take up dissolved nutrients released through modest external digestive activity or obtained from other natural sources, such as carrion, detritus, or organic-rich sea water. It may also be able to accumulate considerable quantities of particulate materials.

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ELECTRICAL RESPONSES TO PHOTIC STIMULATION IN THE EYES AND NERVOUS SYSTEM OF NEREID POLYCHAETES[1]

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The responses of a variety of polychaetes to photic stimulation have been examined by several investigators taking advantage of rather clear-cut, often stereotyped behavior patterns influenced by light. Perhaps the easiest to deal with is the withdrawal reflex on sudden increases and/or decreases in light level characteristic of many tubiculous forms (Nicol, 1950). More complex behavior patterns have been subjected to analysis as in the work of Herter (1926) and Ameln (1930) who studied the behavior of *Nereis diversicolor* under varying conditions of light. With the aid of prostomial eye ablation, they attempted to ascertain the possible differences in the function of the anterior and posterior pairs of prostomial eyes.

Clark (1956), utilizing some species-specific variability in the photoreceptors of *Nephtys,* studied the influence of light on locomotion and orientation in members of that genus. After a consideration of the role of the supra-esophageal ganglion-associated photoreceptors, the suggestion that pigment-surrounded undifferentiated dermal cells may play a part in photo-reception was put forth on the basis of apparent photosensitivity in the posterior part of the worm.

Hauenschild (1961) demonstrated that prostomial eyes are not necessary for the light-controlled triggering of metamorphosis to the heteronereid stage in *Platynereis dumerilii.* Evans (1965) has shown that the withdrawal response to shading is not dependent on the presence of prostomial eyes and the supraesophageal ganglion in *Nereis diversicolor.* Such results make it clear that structures other than prostomial eyes function in photo-reception. These may be unspecialized dermal cells as suggested by Clark (1956), specialized dermal photoreceptors, or photosensitive neurons in the central nervous system. There is ample precedent for neuronal photosensitivity in invertebrates, (Welsh, 1934; Prosser, 1934b; Arvanitaki and Chalazonitis, 1949; Kennedy, 1958, 1960; Yoshida and Millott, 1959) and there is no *a priori* reason to rule it out in polychaetes.

Cells that are presumed to be photoreceptors are known in oligochaetes (Hess, 1925). Similarly constructed cells are known to function in light perception in Hirudinea, (Mann, 1962, for summary; Hansen, 1962; Walther, 1966; Clark, 1966) and are the only obvious photoreceptor equipment in a few polychaetes (*e.g., Nephtys,* Clark, 1956; *Polyophthalmus,* Hesse, 1899). Such cells have not been noted in nereids, although Langdon (1900) described rather complex "spiral organs" she thought to be photoreceptors in *N. virens.* Smith (1957), however, failed to confirm their existence in the species of nereids he studied. None of the simple sensory cells described by him are obvious photo-receptors.

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Work on the electrophysiology of polychaete annelids has been mostly concerned with the functioning of the giant fiber systems (Bullock, 1945, 1948; Nicol and Whitteridge, 1955; Horridge, 1959; Hagiwara, Morita, and Naka, 1964), although aspects of the control of locomotion in *Nereis* (Wilson, 1960), the functioning of mechanoreceptors in *Nereis* and *Harmothoë* (Horridge, 1959, 1963), and the response to mechanical stimulation in *Branchiomma* (Krasne, 1965) have been reported. To date there has been no report of an electrophysiological analysis of any aspect of photoreception in a polychaete, and very few such studies in any annelid (*e.g.*, Prosser, 1934a, 1935; Walther, 1966).

The present report is primarily concerned with the shadow reflex in *Nereis diversicolor* and *Platynereis dumerilii* and the possible role of the dermal and prostomial photoreceptors. Observations on other species are included, where pertinent, for comparison.

A preliminary report of some of the results of this study has appeared elsewhere (Clark, 1966).

MATERIALS AND METHODS

Specimens of *Nereis diversicolor* and *Platynereis dumerilii* were the principal experimental animals, but preparations of *Nereis virens* have also been examined. *N. diversicolor* was collected as required from the banks of the Avon near Bristol. Animals were stored in aerated 50–70% sea water at room temperature or in a refrigerator at 2–4°C. In both cases the worms were provided with glass tubes of a suitable diameter to permit irrigation. Worms handled this way appeared healthy and responsive for at least one week and frequently much longer. *N. virens* was collected at Clevedon on the Bristol Channel and used shortly after collection. Specimens of *P. dumerilii* were supplied by the Marine Station, Millport, and were used within a few days of delivery.

Electrical recording was accomplished in a variety of ways. Electroretinagrams (ERGs) were initially recorded with electrolytically polished stainless steel needles, insulated to the tip, by simply thrusting the electrode through the cuticular lens into the "pupil." This procedure, however, resulted in considerable damage and preparations of this sort did not last more than about one hour. Consequently, most of the ERGs were recorded with glass micropipettes filled with 3 *M* KCl (5–10 megohms resistance in KCl) thrust into the back of the eye after ventral exposure of the supraesophageal ganglion and eyes. Such preparations gave good responses for up to four hours. The amplifier used for the electroretinagrams was a neutralized input capacity amplifier (Bioelectric Instruments, Inc., Type DS2C). Records from the ventral nerve cord, circumesophageal connectives, and segmental nerves were taken either with the previously described steel electrodes or with platinum-irridium hook electrodes. In both cases a Grass P-5 A. C. pre-amplifier was used, with a high impedance input device (Grass, HIP-5) for the steel electrodes. In all cases the results were displayed on a Tektronix Type 502 dual beam oscilloscope and photographed with a Grass C-4 kymograph camera.

The light source consisted of a 6 V, 48 W tungsten filament microscope lamp (Cooke, York) operated from the 6 V laboratory D. C. supply. A shutter was fitted to this which permitted giving light flashes of 1 to 0.01 second duration and could be operated "bulb" or "time" for longer duration flashes. A selenium self-generating photocell intercepted a portion of the beam so that stimulus duration could be monitored on the second beam of the oscilloscope. The lamp housing had provision for introducing filters and a Chance type ON 22 heat filter was kept in place at all times. This prevented any significant temperature rise over the 1–3 hours of the experiments once the preparation had reached room temperature (15–20° C). In no case did the temperature of the bathing medium rise above 20° C, and most experiments were done at 15–17° C.

Light intensity was controlled with neutral density filters (Kodak) and are expressed in terms of per cent transmission of unit intensity (heat filter only) which was approximately 1000 foot candles at the surface of the bathing medium (measured with an S. E. I. Exposure Photometer).

No attempt was made to localize the stimulus to the structure under investigation. This was accomplished in most cases by surgical isolation, and the types of preparations used are described in the text. Anesthesia was not used because recovery failure was a common experience with this material.

Because of the extreme sensitivity of the preparations to mechanical perturbations, it was necessary to "shock mount" the photographic shutter used and to intersperse control shutter operations during the experiments. This was done by operating the shutter and performing all other manipulations with the light source turned off. Such controls always followed the same time course as the experimental procedures. If the preparation responded to shutter vibrations at any time, the run was discontinued until the situation was corrected, and all data previous to the spurious response were discarded.

The medium bathing preparations consisted of 50% sea water in the case of *N. diversicolor* and full strength sea water for the other preparations. This value is well above the point at which *N. diversicolor* from a variety of habitats begins to regulate chloride (Smith, 1955), but information on the regulation of other ions at this salinity level is not available.

Preparations were kept in the dark for at least ten minutes between light flashes unless shorter or longer intervals were appropriate to the response being tested. These other intervals will be apparent in the context of any particular observation.

RESULTS

Responses of the prostomial eyes

An electrode introduced either through the cornea or into the ventral surface of the prostomial eye records an extracellular response to a light flash that is illustrated in Figure 1. This consists of a relatively fast negative-going potential (with respect to an indifferent electrode in the bathing medium), followed by a brief positive deflection, and a slow decay to base line (Fig. 1, A, B). If the preparation is dark adapted for more than 15 minutes, the transient is followed by a "steady-state" potential that is maintained in the light until "off" (Fig. 1C, D). The graded nature of the ERG at different light intensities is shown in Figure 1. If one follows the interpretations of Ruck (1961) of the ERG of the insect ocellus, then the negative components are said to originate in the photoreceptor cell rhabdomere membrane, the positive component in the receptor cell axons. The waveform is thus similar to that seen in a number of arthropod eyes with the exception that spikes are not recorded at this level as they frequently are in arthropod eyes.

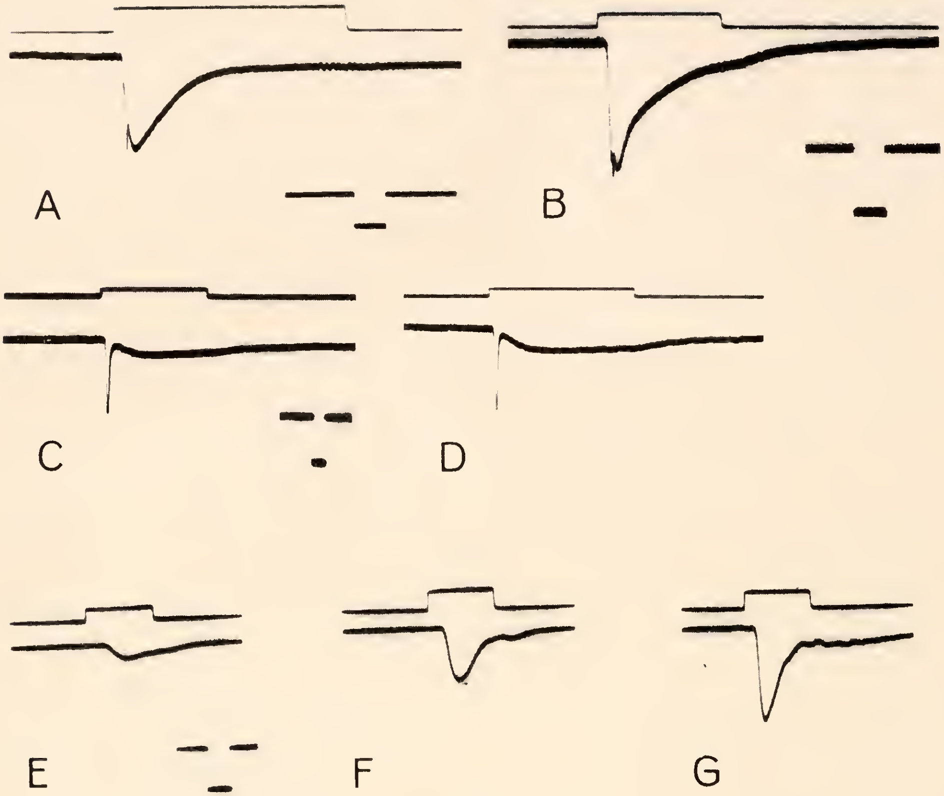

Figure 1. Electroretinagrams from nereid polychaetes. A. and B: Anterior eye of *Nereis diversicolor*. Calibration, 1 mV, 20 ms. C: Posterior eye of *Platynereis dumerilii*. D. Anterior eye of *P. dumerilii*. Calibration for both C and D, 1 mV, 400 ms. E through G: Posterior eye of *P. dumerilii* showing graded response to increasing light intensity; intensity in E = 1% of unit intensity, in F = 10%, in G = 100%. Calibration, 2 mV, 200 ms. In all traces, upward deflection of upper beam indicates "on," downward deflection of lower beam indicates negativity of active electrode. All recordings D. C.

None of the information I have obtained indicates any major consistent difference between *N. diversicolor* and *P. dumerilii* nor between anterior and posterior eyes of the two species.

Attempts to follow the course of dark adaptation were plagued by movement of the preparation, but were finally moderately successful. This could only be judged by a return to a dark-adapted criterion amplitude following a period of light adaptation. If the return to initial amplitude increased with time in the dark, the experiment was judged successful. Using this as a criterion, dark adaptation is approximately two-thirds complete in fifteen minutes, but not complete for ninety minutes or more. Again, no marked difference was noted between species or pairs of eyes. No attempt to vary light intensity or period of light adaptation was made.

There is thus no evidence that any behavioral differences said to be mediated by anterior or posterior pairs of eyes (Herter, 1926; Ameln, 1930) can be ascribed to differences in the properties of the photoreceptors themselves. Such differences are almost certainly centrally mediated, but the rather crude information presented here does not necessarily rule out the possibility that there are differences in the eyes other than the obvious positional ones.

Responses recorded from the nervous system.

A. Supra-esophageal ganglion It is possible to record an inverted (positive-going) "electroretinagram" from the supra-esophageal ganglion. Destruction of the eyes abolishes this response, so it is assumed to originate in the prostomial eyes.

Using steel electrodes, spike potentials have been detected in the brain at "off," but records are so inconsistent that it is impossible to assign them to eyes or possible prostomial dermal photoreceptors.

B. Circumesophageal connectives Records taken with external hook electrodes from the circumesophageal connectives show no activity clearly associated with "off"

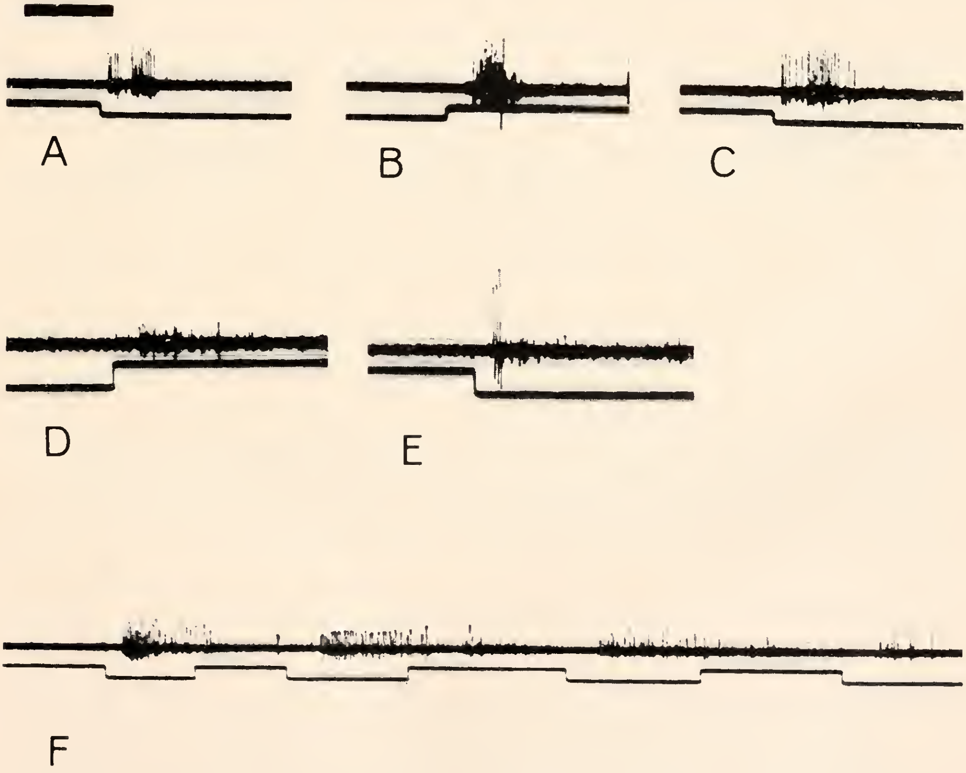

FIGURE 2. Recordings taken with steel electrodes from the ventral nerve cord of *N. diversicolor*. A–C: sub-esophageal ganglion. D: "on" response from cord posterior to sub-esophageal ganglion in a headless *N. diversicolor*. E: "off" response in the same preparation. F: recording posterior to a cut in the ventral nerve cord in the posterior half of *N. diversicolor*. Time calibration in A = 500 ms and applies to all records.

or "on." On a few occasions the impression was gained that slow build up in activity followed "on," but there was so much "spontaneous" activity that was so variable it was impossible to make a clear association. On the other hand, large fiber activity was easily demonstrable by mechanical stimulation of the prostomial antennae indicating at least some functional pathways. On a few occasions, bursts of activity at "off" were detected by steel electrodes thrust into the circumesophageal connective, but large external electrodes failed to detect this.

These results indicate that the two kinds of information are transmitted over different pathways to the ventral nerve cord where both activate the giant fibers. This would also seem to provide some evidence that different giant fibers may be activated by the different sensory modalities. The circumesophageal activity recorded on mechanical stimulation is clearly giant fiber activity and as the lateral giant fibers are the only ones found in the circumesophageal connective (Nicol, 1948; Smith, 1957), it is almost certainly this that is recorded. As the median giant fiber is also activated by anterior stimulation there are either separate fibers serving as input to the median fiber (terminating in the sub-esophageal ganglion) or there is cross-over between the two. Since the lateral giants have the higher threshold to mechanical stimulation (Bullock, 1945), the system consists of either separate pathways or a synapse to the lateral giants that requires spatial and/or temporal summation. In any event, it seems clear that the anterior terminations of the lateral giant fibers are not activated via the anterior photoreceptors.

C. Subesophageal ganglion If the head and circumesophageal connectives are left intact and the ventral nerve cord posterior to the subesophageal ganglion is intact, the activity reproduced in Figure 2A, B, may be recorded from the subesophageal ganglion. There is a burst of fine fiber activity at both "off" and "on." Severing the ventral nerve cord just posterior to the subesophageal ganglion does not appreciably affect this activity (Fig. 2C). Under these circumstances there is still no indication of giant fiber activity, but it does show that photic information is transmitted from the anterior part of the worm, presumably via the circumesophageal connectives, but possibly also from other sensory input, directly to the subesophageal ganglion. The difference in latency between the "off" and "on" responses may simply be due to different transmission velocities in the internuncial fibers responding to the two conditions.

D. Ventral nerve cord If records are taken from any point in the ventral nerve cord there is almost always a fine-fiber response at "on" (Fig. 2D), and, if the animal has been left in the light for more than 30 seconds, a giant fiber response at "off" *if the anterior part of the worm is included in the preparation* (Fig. 2E). If the nerve cord is severed at any point posterior to about the middle of the worm and the recording taken from posterior to the cut, there are both "on" and "off" responses, but these are fine fiber responses. Transferring the electrode anterior to the cut records giant fiber activity at "off" in the same worm. That the giant fibers (paramedials) activated by posterior afferents are still functional and the electrode will record their activity is easily demonstrated by mechanical stimulation of the posterior end of the worm. This indicates that photoreceptor input to the giant fiber system is limited to, at most, the anterior half of the worm, but also that photoreceptors are present posteriorly that activate only the fine fiber system (Fig. 2F).

If the head (prostomium) of the worm, or, indeed, the first several segments are removed, the same cord responses are obtained, proving that the prostomial eyes are not necessary for the giant fiber response. The only difference consistently apparent in the cord responses of intact and headless worms is an increase in latency of the giant fiber response (recording from the same site in the same worm) in the headless animals (Fig. 3A and B). Three explanations for this phenomenon seem possible. 1. The giant fiber response is normally triggered by the eyes which

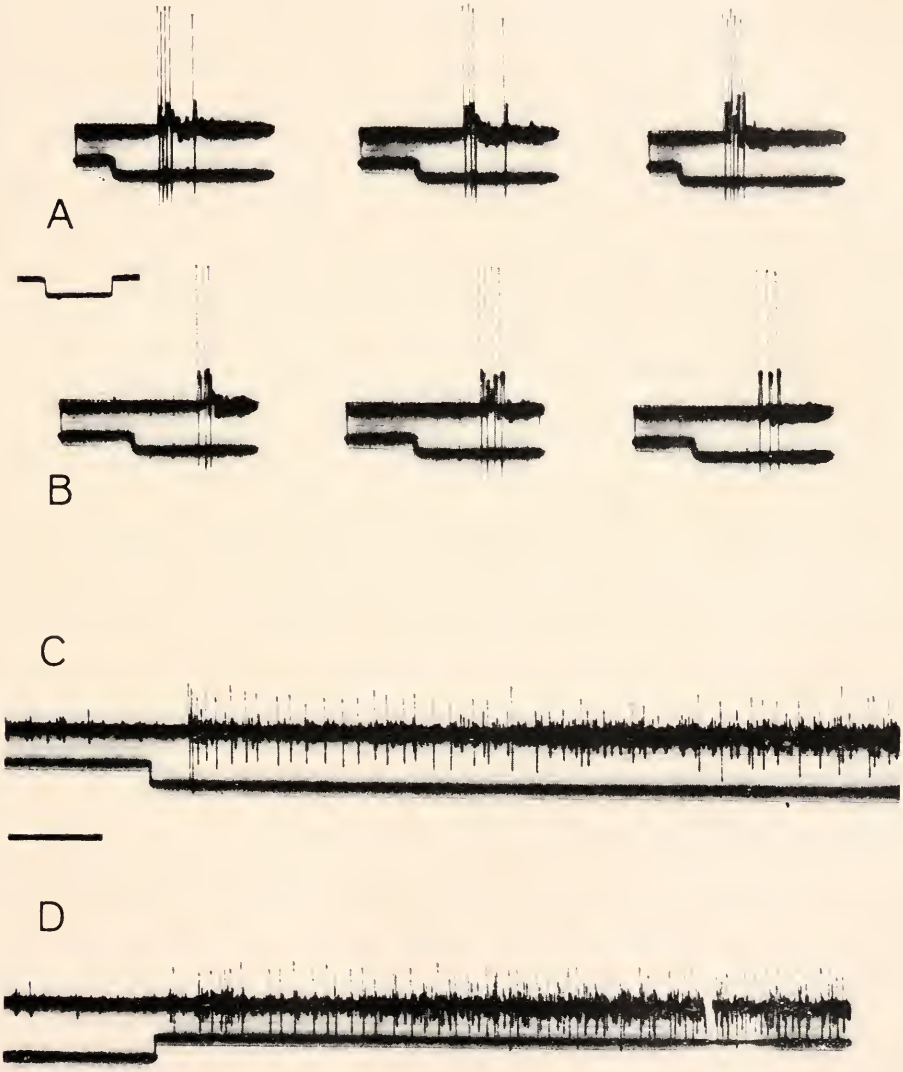

FIGURE 3. A, B: records taken from the same location on the ventral nerve cord in the same preparation of *N. diversicolor*. A, with head intact; B, headless. Time calibration = 150 ms. C: "off" response in segmental Nerve II, *N. virens*. D: "on" response in the same preparation. Time calibration = 500 ms.

have a more direct connection to the giant fiber system and hence a shorter latency; 2. The response is due to temporal and/or spatial summation of the dermal photoreceptor input and removal of the head removes many dermal photoreceptors leaving more scattered, less directly-connected receptors to serve as the trigger; or, 3. it is an injury effect. The last explanation could not be definitely ruled out, but the longer latency persisted unchanged for at least an hour after head removal.

Another aspect of the giant fiber response should be mentioned. In many of the records it is clear that more than one type of giant is firing, and in some only one type, but repetitively. If shadows are delivered in succession, the first few will show multiple firing, but the record soon shows activity from a single giant fiber, always the smaller if two amplitudes were previously apparent. A possible interpretation of this observation is that a shadow causes one or a series of spikes in the median giant fiber. This in turn triggers a contraction, and as soon as the worm begins to move there is ample input from, *e.g.*, bristle receptors to trigger more median spikes and at times, the lateral giant fibers. Consequently, as long as there is movement in the worm the sensory input is not restricted to that from the photoreceptors.

E. Localization of dermal photoreceptors It was first established on several specimens that the nerve cord itself was not photosensitive to the extent that it gave any of the responses noted above. Isolated nerve cords showing good spontaneous activity and nerve cords *in situ* but with all segmental nerves cut showed no change in activity at "on" or "off." However, as long as there was some connection via segmental nerves to the anterior body wall and parapodia, a giant fiber response could be obtained at "off." The smallest fragment actually recorded from that gave this response was made up of seven segments.

Parapodial removal in headless (prostomium and peristomium removed) worms abolished the response, but if the head was intact the giant fiber response always occurred, even with the parapodia removed. Dorsal body wall removal alone did not abolish the response. From this it was concluded that the dermal photoreceptors are widely distributed on the prostomium and the peristomium, and the parapodia. They may be present on the dorsum, but it is certain that they are not limited to that region. The fact that parapodial removal abolished the response in headless worms may also be taken to prove that the photoreceptors are not located ventrally, for parapodial removal would not disrupt ventral sensory input. I have not attempted to localize the receptors more precisely by removing bits of the parapodia in turn, but the possibility that they are limited to, *e.g.*, cirri, exists. In connection with these ablation experiments, the "spiral organs" described by Langdon (1900), have the following distribution: outer and lateral surfaces of the palp bases; outer surfaces of bases of cephalic cirri; dorsal surface of the prostomium; the first setiger on both the dorsal and ventral surfaces. The number located mid-dorsally decreases posteriorly. They are also found on the tips of the gill lobes of each parapodium. In general, the number is related to body diameter and so decreases posteriorly. This distribution fits fairly well with the observed activity, but the fact that these complex organs have not been seen by other workers (*e.g.*, Smith, 1957) casts doubt on the possible causal relationship. There is no other single type of sensory structure (*i.e.*, different from the others) that has been described to have a distribution that would explain the results.

F. Segmental nerves If the body dermal photoreceptors are located principally on the parapodia (as the evidence indicates) one would expect to be able to record sensory input in segmental Nerve II in response to light stimuli. Attempts to record this activity have been unsuccessful despite considerable effort. I have been able to record sensory input from a variety of mechanoreceptors, but there is no discernible response to light or shadows. Horridge (1963) has shown that peripheral sensory convergence is unlikely by demonstrating that Nerve II has a few large axons identified as mechanoreceptor afferents and a few thousand small ones. This makes it possible to surmise that the photoreceptor axons are small and thus difficult to record from in a nerve bundle that is dominated by the mechanoreceptor response.

Records from the central stumps of Nerve II in *N. virens* show bursts of activity at "off" and at "on" (Fig. 3C, D). This is also true of Nerves I and IV, although I have obtained very few records from the latter two. I have not succeeded in recording any activity from Nerve III. Records from Nerve II in *N. diversicolor* are similar to those shown for *N. virens.* The long persistence of activity in Nerve II does not correspond to cord activity where the responses to light and shadow are relatively brief. The continued activity may be due to the stimulation of movement in the preparation. The fact that the response in the parapodial nerve occurs at both "off" and "on" in *N. diversicolor* indicates that it is not due to the giant fibers, for they do not fire at "on" in that species. Further evidence of this is seen in the parapodial "pointing" reflex. If *N. diversicolor* is mechanically stimulated anteriorly, the worm withdraws its head and all of the parapodia are swung in toward the body pointing forward. If the tail is stimulated, it is withdrawn and the parapodia point backward. If the light intensity is lowered (shadow) the forward pointing reflex is evoked; if the light intensity is increased, the backward pointing reflex follows. The latter condition does not generate giant fiber activity, but can still lead to the useful response of tail withdrawal.

G. Habituation Several reports on habituation to stimuli in nereid polychaetes have appeared (Clark, 1960a, b; Evans, 1965; Evans, 1969a, b; Clark, 1966 for summary). These reports establish that the habituation process seen at the behavioral level is not a simple one. It appears that habituation to any one narrowly defined stimulus is straight-forward, but if two kinds of stimuli are interspersed, or if there is some slight change in the way a stimulus is presented, the time course of the process may be altered, and in some cases the response enhanced (Clark, 1960b, 1966).

In this study, the number of trials to failure of the giant fiber system was many fewer than that of the fine fiber response. If the light was turned off every thirty seconds for 2–3 seconds the giant fiber response failed after a maximum of 25 trials. If the intertrial period was reduced to 5 seconds, the giant fibers failed after 1–4 trials. In both cases fine fiber responses continued for as many as 56 trials with 30 second intervals and 20 trials at 5 second intervals. In neither case were fine fiber responses taken to extinction.

In the behavioral work it was impractical to make a distinction between fast and slow withdrawal after the first few responses, but a difference between head and tail withdrawal has been observed. Tail withdrawal is slower than head withdrawal, and the former occurs at light "on," the latter at light "off" (Evans, 1969a). One cannot say the fine fiber activity generates tail withdrawal and giant fiber activity head withdrawal in any general sense, but in the response to photic stimulation one can say that any known response involving the giant fibers is always correlated with head withdrawal. The fine fiber response, on the other hand, is not necessarily correlated only with tail withdrawal.

The fact that fine fiber responses persist after the giant fiber response is extinguished indicates that the site of habituation for the latter is at some point on the sensory side, perhaps at the sensory-to-giant synapse. Failure of the worm to respond after less than 40 trials (Evans, 1969a) indicates that a second point of failure is on the motor side of the central nervous system because the fine fiber central activity persists beyond this point.

Attempts to demonstrate re-sensitization of the giant fiber response by applying a mechanical stimulus after giant fiber failure were unsuccessful.

Discussion

The results of this study provide no evidence for any difference in the two pairs of prostomial eyes as far as the sensory event is concerned. Any behavior difference that might be ascribed to one pair or the other is probably due to central processing or to the position of the eyes. The anterior eyes are directed forward and up, the posterior pair laterally and up, and it is thus quite likely that they are stimulated differently.

The wave-form of the electroretinagrams presented are not markedly different from those seen in, *e.g.*, insect ocelli recorded under similar conditions. Spikes are not seen, however, and this may indicate that the photoreceptor cells themselves do not spike, but certainly does not prove it. Intracellular recording may be expected to yield good evidence on this point, but that has not been possible with this material.

The brief positive "notch" on the leading edge of the ERG (Fig. 1A, B) may be due to the passive spread of an axonal event as Ruck (1961) has suggested for insect ocelli, but it may be indicative of a regenerative event such as that seen in *Limulus* retinular cells (Benolken, 1961; Benolken and Russell, 1966). Again, however, intracellular recording is required for substantiation of this.

The ventral nerve cord responses offer satisfying electrical correlates to some aspects of known behavior, present some puzzles, and uncover new information not readily obtainable with behavioral techniques. Most of this information can be applied only to *N. diversicolor* with confidence.

Fast head withdrawal and the forward-pointing parapodial reflex is associated with a *decrease* in light intensity behaviorally, and this stimulus generates a giant fiber response in the central nervous system. An *increase* in light intensity usually leads to relatively slow tail withdrawal and the backward-pointing parapodial reflex (Evans, 1969a). Under these conditions only a fine fiber response is seen in the cord. Further, if the anterior part of the worm is excluded, giant fiber responses are not seen either at "on" or "off" which suggests that a tail withdrawal response to photic stimulation of the posterior part of the worm is not mediated by the giant fibers. A system of this kind places the emphasis on saving the head which is the structure that is normally out of the burrow during feeding and thus in greatest jeopardy. On those occasions when the tail becomes exposed, the increase in illumination would be sensed and lead to tail withdrawal. It would also seem that if only the tail was exposed, and for some reason was not retracted into the burrow, a sudden decrease in light intensity would also elicit tail withdrawal. Under the usual laboratory conditions for examining behavior in intact animals this event is seldom seen because the giant fiber response generated in the anterior part of the worm would override the posterior "off" event. The advantage of preserving the head for purely sensory and feeding reasons is obvious, but it is also the case that in *Nereis* heads cannot be regenerated while tails can (Cassanova, 1955).

The evidence that the prostomial eyes alone are capable of generating a giant fiber response is ambiguous. There was no satisfactory way of ablating all possible dermal photoreceptors to test this. It is clear, however, that the prostomial eyes are not necessary for the response. The only apparent effect of eye removal (which in the observations reported involves at least removal of the prostomium as well) is an increase in latency of the giant fiber response. This may indicate the eye contribution, but could just as well be the effect of brain and prostomial dermal photoreceptor removal.

The absence of activity in the segmental nerves associated with light regimen is somewhat puzzling in view of the ablation experiment results. Horridge's (1963) observations of the morphology of the segmental nerves offer the most likely explanation.

The efferent activity in the segmental nerves is about as one might expect for normal motor discharge to the parapodia and body wall muscles. This study does not distinguish between possible different kinds of motor output (slow, fast, inhibitory), nor does it distinguish between motor and efferent sensory activity as described by Horridge (1963). The correlation with the light regimen is satisfying, but not unexpected.

The contribution this study makes in the explanation of habituation is to suggest the possible points of synaptic failure by demonstrating that neural activity persists for a greater number of trials than does behavioral activity. This result is of limited significance because considerable variability is quite likely a characteristic of these events and many more examples are needed to establish the relationship.

The hospitality of the late Professor J. E. Harris (subsequently Vice-Chancellor), Department of Zoology, University of Bristol, is gratefully acknowledged. Professor R. B. and Dr. M. E. Clark were particularly helpful in all phases of the work in England, and in making us feel welcome and ensuring a most enjoyable and profitable sabbatical year. All members of the Zoology Department at Bristol were considerate and friendly, and they have my sincere thanks.

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SUMMARY

1. Anterior and posterior pairs of prostomial eyes in *Nereis diversicolor* and *Platynereis dumerilii* display similar electroretinograms.

2. Electrical responses recorded from the ventral nerve cord elicited by changes in the level of illumination with and without prostomial eyes are as follows: (a) Prostomial eyes alone may be capable of triggering a giant fiber response at "off." (b) Ablation of prostomial eyes leads to a longer latency in the giant fiber response but does not abolish it. (c) In the absence of prostomial eyes a giant fiber response is elicited at "off" only if the light level change involves the anterior part of the worm. (d) Fine fiber responses at both "off" and "on" occur in the absence of prostomial eyes when the change in illumination occurs at any place on the body of the worm.

3. Localization experiments indicate that dermal photoreceptors are located primarily on the pro- and peristomium and on the parapodia.

4. Attempts to habituate the cord responses indicate that the site of habituation for the giant fiber response is on the sensory side of the pathway.

5. The significance of the different responses to illumination changes (fast head withdrawal, slow tail withdrawal) in relation to the mode of life of the worm and its powers of regeneration are discussed.

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EVIDENCE FOR THE ENDOGENOUS CONTROL OF SWIMMING IN PINK SHRIMP, *PENAEUS DUORARUM*[1]

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Most animals habitually exposed to water currents orientate and swim in either an upstream or downstream direction. Changes in physical or chemical conditions may elicit a reversal of this rheotactic response. In a few studies (Beauchamp, 1933, 1937; Keenleyside and Hoar, 1954; Creutzberg, 1961) these reversals have been shown to be adaptive, enabling the movement or displacement of the animal in a direction advantageous to it.

Such reversals have been found in pink shrimp, *Penaeus duoarum,* Burkenroad (Hughes, 1969). Juveniles of this species usually swim actively against a current. However, when salinity is reduced, when the animals are starved, or when the water becomes polluted, the shrimp will turn about and swim downstream, with active swimming often giving way to passive drifting. The normal rheotactic response enables juvenile shrimp to evade inshore displacement by flood tides while the reversal, occurring in response to decreased salinity, enables them to utilize ebb tides to effect offshore movement at an appropriate stage in their life cycle.

During the course of the above study, it became evident that all individuals of a group, maintained within a constant current in the laboratory, would, at any one time, carry out essentially the same type of swimming in terms of its velocity and direction with respect to current. Yet the velocity and direction of swimming of the group as a whole often varied throughout the night, despite the absence of any change in external conditions. This indication of endogenous control over swimming was further investigated.

Apparatus and Methods

The experiments were conducted in two identical ring-shaped "current chambers" constructed of "Plexiglas" and based on the design used by Creutzberg (1961) for his study of migration of elvers. This design has been described and figured elsewhere (Hughes, 1969). The apparatus was housed in a light-tight enclosure in the laboratory and illuminated constantly by a centrally placed 5w red bulb, which provided just enough light to permit observation at night. In addition, a 150 w flood lamp, deflected off the white ceiling, supplied the "daytime" illumination during light cycles which approximated those in nature. The salinity of the water in the chambers was usually between 32 and 34‰. No fluctuations during experiments was possible since the water was circulated through a closed system. Temperature within the laboratory was maintained constant throughout this work. The current speed, maintained at 12 cm/sec, was arbitrarily chosen as one against which shrimp were able to swim and which would not disturb the sandy substrate.

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On the night preceding each experiment, juvenile shrimp (total length, 6–10 cm) were collected from the ebb tide in a channel within the Everglades estuary of southern Florida, where tides are semidiurnal. They were collected from different times of the lunar cycle, and thus from tides occurring at different times during the night. [This species is only active at night (Hughes, 1968).] From there they were transported immediately to the laboratory and placed in the current chambers. Seven were used at a time during the experiments of series I (Fig. 1), and eight at a time in the experiments of series II (Fig. 2).

During "daylight" hours the shrimp remained buried within the substrate but, after the floodlamp was extinguished in the evening, they all invariably emerged within 20 minutes. Their movements were recorded at intervals from this time until sunrise the following day when the enclosure was again illuminated, and those shrimp, which had not already done so, reburrowed. Movements were recorded in terms of the number of shrimp which passed a vertical mark on the current chamber in either an up or downstream direction during a two minute period. Being a circular chamber, the same animal would often be counted several times during each count period. [The extent of movement with the current per unit of "effort" is obviously greater than movement against the current. Therefore to enable more ready comparison of the two activities, up and downstream movements have been plotted on different scales (Figs. 1 and 2).]

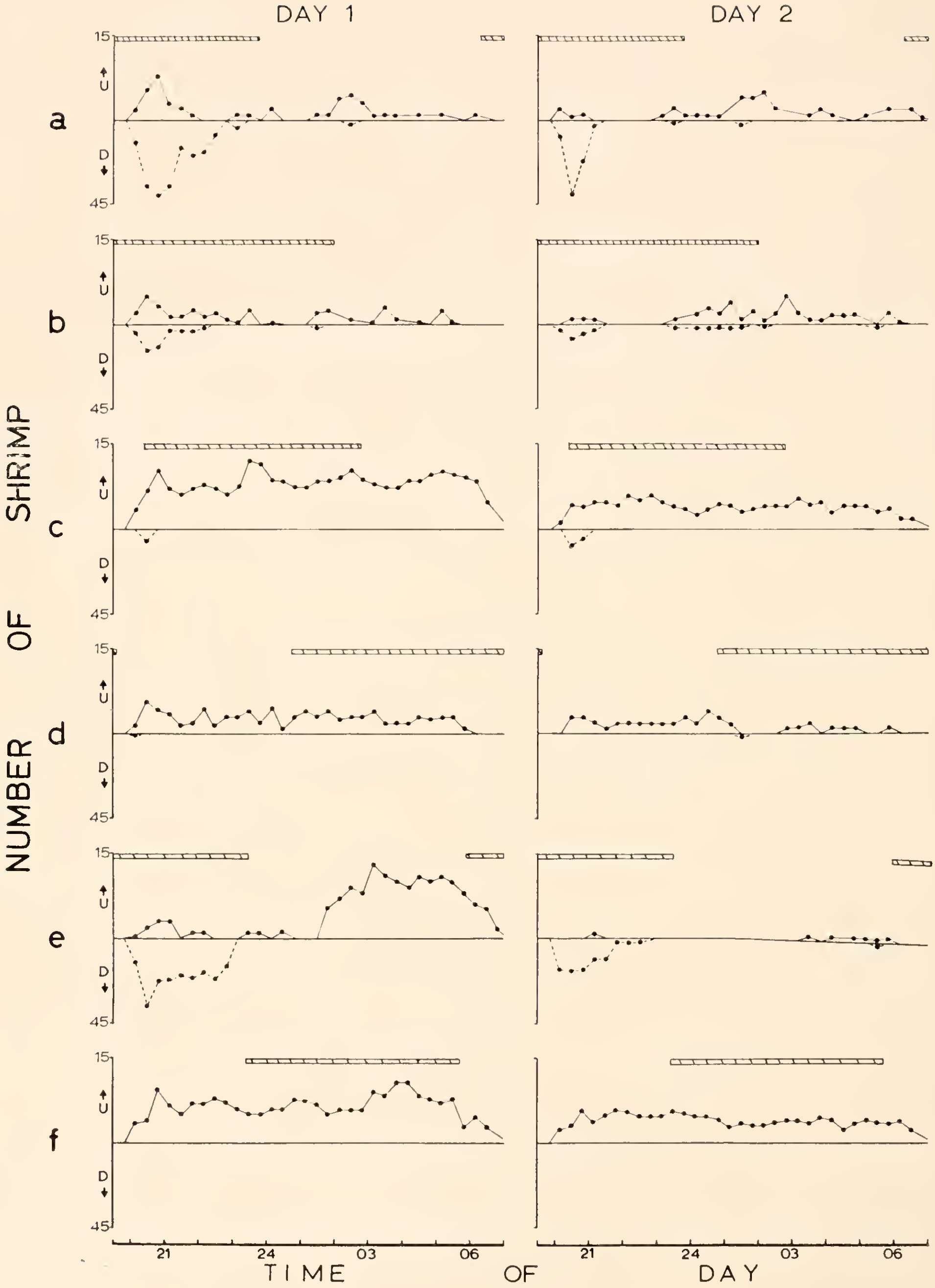
RESULTS

In series I (Fig. 1) only one chamber was used and the movements of the shrimp were recorded on two consecutive nights following their capture. In series II (Fig. 2) similar groups were placed in two identical current chambers and the movements of both groups were recorded on only one night following capture.

A marked synchrony in the activities of all or most individuals was evident from observations of their movements within the current; generally all shrimp within a group would, at the same time, carry out essentially the same type of swimming, in terms of their velocity and direction with respect to current. Although social facilitation may play a role in maintaining this synchrony, observations of the shrimp themselves suggested that such a role was small. At times when direction of swimming with respect to current was reversing, those shrimp which had already changed their direction of swimming had no noticeable effect on others, swimming in the opposite direction, despite frequently colliding with them. Indeed, few external stimuli modified their swimming. If fed during either intense downstream or upstream swimming the shrimp would usually hold and eat the food while continuing to swim in the same direction as before.

Although the results sometimes indicate that both upstream and downstream swimming occurred concurrently, individual shrimp were not in fact continuously swimming in different directions but all or most were swimming upstream, releasing the substrate and drifting downstream before settling and resuming upstream movement.

In addition to the synchrony existing among individuals, the results indicate a similarity between (i) the swimming of a group of shrimp within the current chamber on both the first and second night following its collection from nature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(Fig. 1), and, (ii) the swimming of two groups, collected together but maintained in separate current chambers (Fig. 2).

It is also apparent that the general pattern of swimming, especially with regard to its direction, is influenced by the time of the ebb tide from which the shrimp were collected in nature. In this connection a valid generalization appears to be that, those shrimp which, on the day of their collection, emerged from the substrate into an ebbing tide, would, the following day in the current chamber, swim downstream for a period approximating the ebb in nature (Fig. 1a and e; Fig. 2b and c). However, those which emerged into a flooding tide would swim upstream throughout the following night in the current chamber (Fig. 1c, d, and f; Fig. 2d). The behavior of those which emerged into the end of one or the other tide or into the slack water between the tides was more ambiguous (Fig. 1b and c; Fig. 2a).

The relationship between the tide cycle from which the shrimp were collected and their subsequent swimming in the current chamber was investigated on four other occasions, twice with shrimp which had emerged from the substrate into ebb tides and twice with shrimp which had emerged into flood tides. Their swimming during the first few hours of the evening was intermittently observed and each case further confirmed the above generalizations.

Discussion

The sum of these results is evidence that both direction and level of swimming activity is under some measure of endogenous control. The predictable relationship existing between the stage of the tidal cycle in nature and the pattern of swimming in the current chamber suggests that some aspect of the tidal cycle to which the animals were exposed immediately prior to capture, entrains a pattern of swimming which may persist for at least two days in a current, constant in terms of speed and "water quality." From these results the nature of the timing mechanism and its entraining factors is obscure. Salinity changes are, however, suggested as being of possible significance. The direction of swimming of juveniles with respect to current may be directly influenced by their responses to changes in salinity (Hughes, 1969). These changes in salinity and consequent reversals in the direction of swimming occur regularly with change of tide in nature. Earlier experiments indicated that shrimp responded more readily to a salinity decrease imposed at the time of the ebb tide than to one imposed at the time of the flood (Hughes, 1967). This suggested that salinity change was the probable Zeitgeber maintaining the synchrony between the pattern of swimming and the tide cycle. Subsequent experiments were often contradictory and gave no unequivocal indication of a "periodically changing sensitivity of the organism to the stimuli of the Zeitgeber" (Aschoff, 1965, p. 95). It is therefore no longer possible to conclude that salinity change is the entraining factor. In addition, the absence of downstream swimming

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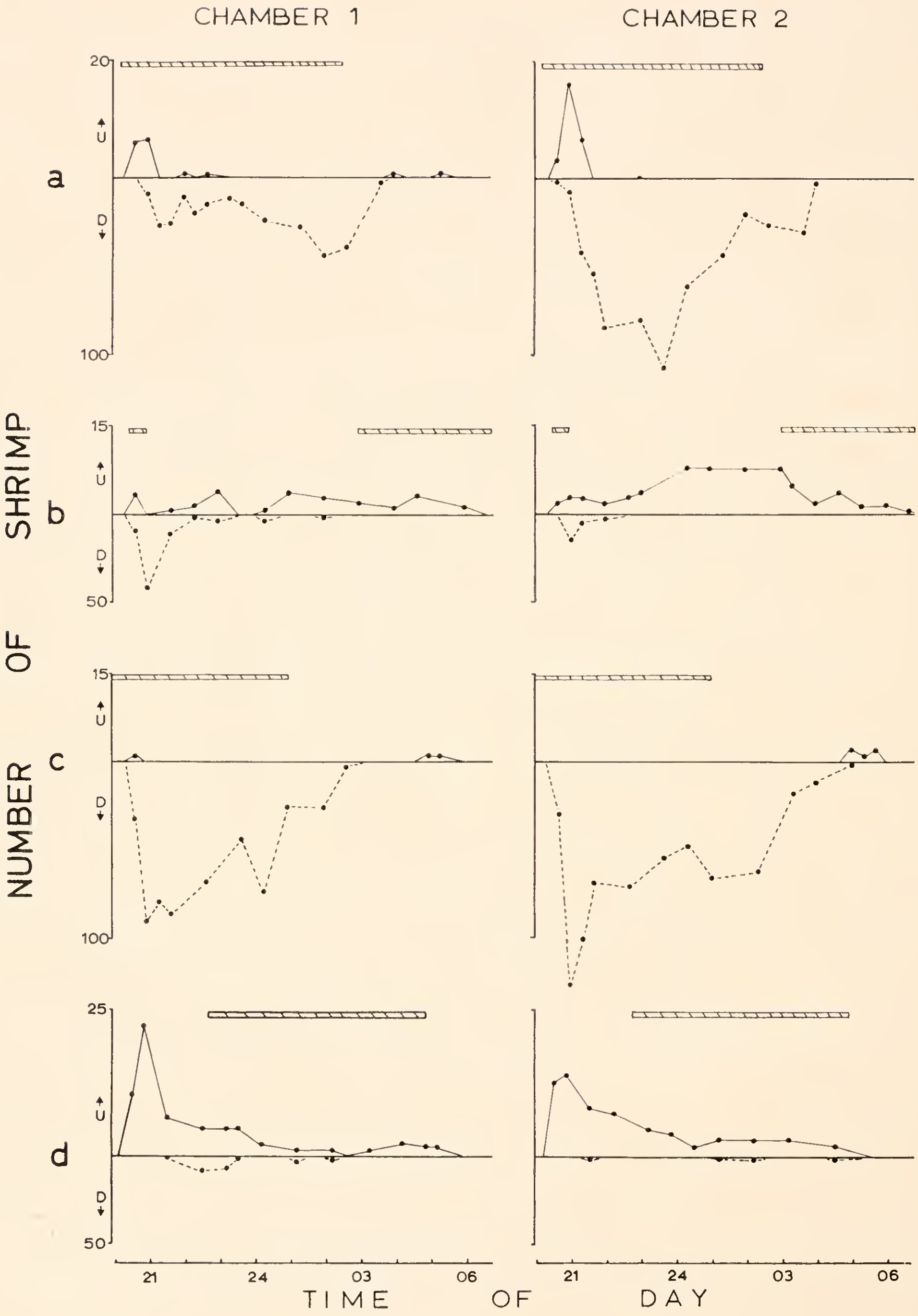
Figure 1. The number of shrimp which moved in either an up or downstream direction during observations made at 10 or 20 minute intervals in a current chamber throughout the two nights following their collection from nature. Upstream movement (U) is indicated by the points connected by the unbroken line above the abscissa, and downstream swimming (D) by the broken line below the abscissa. The six pairs of "curves" (a–f) represent the records of shrimp collected less from ebb tides occurring at different times of the night (late summer 1966). The time of the ebb tide on the night of capture is indicated by the transverse bars.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in the current chamber, during the time of late night ebb tides, makes any explanation, solely in terms of a tidal rhythm, difficult.

The occurrence of spontaneous reversals in rheotaxis has apparently not been observed before. In this case the reversals were apparently linked adaptively with the tide cycle: downstream swimming in the current chamber occurred only at the time of ebb tides in nature. It did not, however, occur at the time of all ebb tides but only those occurring early in the evening. In nature the response of the shrimp to the salinity decrease accompanying the ebb tide would ensure that shrimp swim downstream (and thus move in an offshore direction) during all ebb tides.

Juvenile penaeids often carry out extensive movements between their inshore "nursery areas" and the offshore waters in which they spawn. There is evidence that they school during these movements and that some cohesion of the aggregations is maintained. This probably ensures that individuals at a similar developmental stage will arrive at spawning sites together and it may confer a measure of protection from predators. The method whereby cohesion is maintained in pink shrimp, especially in view of their nocturnal activity, is not clear. However, the marked similarity of the swimming behavior between groups and its endogenous control, as shown by these results, further supports the contention (Hughes, 1968) that cohesion of aggregations of migrating shrimp may largely be maintained by the control over their activities of various biological timing mechanisms. Indeed, there appears to be no evidence for the presence of any form of social interaction which could similarly serve this purpose.

I would like to thank the Committee for Research and Exploration of the National Geographic Society, Washington, D. C., for their generous support of this project. I also thank Dr. C. P. Idyll, of the Institute of Marine Sciences, University of Miami, for providing facilities within the Division of Fishery Sciences, and Drs. H. Schöne and K. Hoffmann of the Max-Planck-Institut für Verhaltensphysiologie, for their comments on the manuscript.

Summary

1. Evidence that the swimming of migrating juvenile pink shrimp is under some measure of endogenous control was derived from experiments which indicated (i) that the pattern of swimming exhibited by a group of shrimp, maintained under constant conditions within a current of water, was similar over each of the two nights following their collection from nature, and (ii) that the swimming of two such groups, collected together, but maintained in separate current chambers within the laboratory, was similar during the night following their capture.

2. Endogenous control over swimming extended to the sign of rheotaxis which, during certain nights, in the absence of change in external conditions, would reverse in all shrimp at approximately the same time.

FIGURE 2. The number of shrimp in two separate but identical current chambers which moved in either an up or downstream direction during observations made at intervals throughout the night following their collection from nature. The records were repeated on four occasions (a–d) with groups of shrimp collected from ebb tides occurring at different times of the night (mid-summer 1967). For further explanation see Figure 1.

3. A predictable relationship occurred between the tide cycle to which the shrimp were exposed prior to capture and their subsequent swimming in the laboratory. The adaptive nature of this relationship is suggested from the fact that downstream swimming, which in nature occurs only during ebb tides and facilitates the offshore movements of juveniles, occurred in the laboratory only at the time of ebb tides in nature. It did not, however, occur at the time of all ebb tides but only during those occurring early in the evening.

4. It is suggested that the cohesion of the aggregations of migrating shrimp may largely be maintained by means of the synchrony imposed on the activities of all individuals by endogenous timing mechanisms.

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OBSERVATIONS ON THE NUTRITION OF SEVEN SPECIES OF RHYNCHOCOELAN WORMS[1]

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Such information as is available concerning nutrition within the phylum Rhynchocoela is largely restricted to reports on the food and feeding mechanisms of relatively few species. The group, which is predominantly free living in habit, is generally regarded as carnivorous or scavenging, and potential food is detected either from a distance by means of chemotactic receptors (Reisinger, 1926; Coe, 1943; Beklemishev, 1955), or at short range by the eyes (Jennings, 1960; Roe, 1967). Active living prey are caught by the proboscis; in the hoplonemerteans stylet bulb secretions frequently cause paralysis or death of the captured organism, but no comparable effect is reported in the palaeonemerteans, heteronemerteans or bdellonemerteans, which lack proboscis armature. Partial proboscis retraction then brings food to the mouth, where it is either swallowed intact or sucked dry of its softer body parts. In contrast, inactive living prey or decaying food materials are ingested directly without prior proboscis eversion (McIntosh, 1873–1874; Du Plessis, 1893; Reisinger, 1926; Coe, 1943; Gontcharoff, 1948; Hylbom, 1957; Tucker, 1959; Jennings, 1960; Hickman, 1963; Roe, 1967).

Digestion has been variously reported as very rapid (Wilson, 1900; Child, 1901; Piéron, 1914; Coe, 1943; Gontcharoff, 1948; Jennings, 1960, 1962a), or as lasting several hours or even up to a few weeks (Du Plessis, 1893; Coe, 1943; Beklemishev, 1955). Reisinger (1926), using histological methods, showed that in the hoplonemertean *Prostoma rubrum* an extracellular proteolytic phase is followed by intracellular proteolysis and lipolysis, with carbohydrases playing only a minor role. Jennings (1962a), using histochemical methods, showed that in the heteronemertean *Lineus ruber* the extracellular phase is acidic and involves cathepsin-C type endopeptidase. This is followed by intracellular completion of digestion by exopeptidases, lipase and carbohydrases.

In contrast to these descriptions of digestion in carnivorous species, Gibson and Jennings (1969) showed that in the entocommensal bdellonemertean *Malacobdella grossa*, an atypical microphagous species with a large proportion of plant material in its diet, the complement of proteolytic enzymes is very much reduced and digestion effected primarily by carbohydrases.

Little is known concerning food reserves in the Rhynchocoela. Only three species have been examined and in all of these fat forms the principal reserve, supplemented by smaller amounts of glycogen (Reisinger, 1926; Jennings, 1960; Gibson and Jennings, 1969).

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[1] Supported by research grant AI-06295 of the United States Public Health Service.

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It can be seen from this brief review that no comparative account of nutrition within the phylum exists. In the present study, therefore, the diet, feeding mechanisms, digestive physiology and food reserves of seven species representative of the three major orders of rhynchocoelans have been investigated, in order to establish the general pattern of nutrition within the phylum.

MATERIALS AND METHODS

The following rhynchocoelan species, listed systematically, have been examined:

ANOPLA
  Order PALAEONEMERTINI
    Cephalothrix bioculata Oersted
    Cephalothrix linearis (Rathke)
  Order HETERONEMERTINI
    Lineus ruber (Müller)
    Lineus sanguineus (Rathke)
ENOPLA
  Order HOPLONEMERTINI
    Amphiporus lactifloreus (Johnston)
    Tetrastemma melanocephalum (Johnston)
    Prostoma rubrum Coe

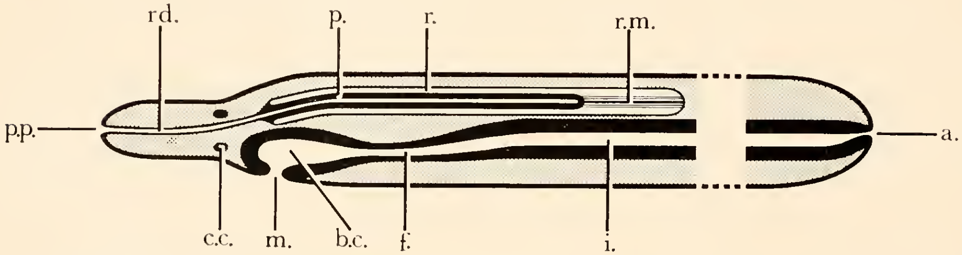


FIGURE 1. *Cephalothrix bioculata.* Schematic vertical longitudinal section to show the arrangement of the alimentary canal and proboscis, characteristic of the Palaeonemertini. a., anus; b.c., buccal cavity; c.c., cerebral commissure; f., foregut; i., intestine; m., mouth; p., proboscis; p.p., proboscis pore; r., rhynchocoel; rd. rhynchodaeum; r.m., retractor muscle of proboscis.

Prostoma rubrum, the only freshwater species investigated, was obtained from ponds near Stratford, Connecticut through the courtesy of Dr. John J. Poluhowich. The other species were collected from the intertidal zones at Filey Brigg and Robin Hood's Bay, on the Yorkshire coast.

For general histological examination specimens were fixed in marine Bouin, Susa, or 10% neutral formalin. Excessive contraction and coiling during fixation was prevented by using these fixatives at 37° C and in some instances prior relaxation in 8% aqueous magnesium chloride was also found to be advantageous. Paraffin sections cut at 6 μ were stained by routine methods, including hematoxylin and eosin, Feulgen, Mallory's trichrome, Mayer's haemalum, Alcian blue (for mucins), the bromphenol blue method of Mazia, Brewer and Alfert (1953) for proteins, and the periodic acid-Schiff (PAS) method for mucins and carbohydrates.

The nature of the diet was investigated from sections and squashes of the gut of freshly collected specimens, and from preference tests in which each species was presented with representatives of the fauna associated with it in nature. Mechanisms for capturing and ingesting the chosen food were studied by direct observation, particular attention being paid to the condition and type of food that evoked proboscis eversion and to the times required for complete ingestion. Steinmann's fluid was used in many cases to fix specimens in the act of feeding, this fixative acting rapidly enough to prevent proboscis withdrawal or muscular contractions.

The site and sequence of digestion were studied by fixing series of individuals at progressive intervals after an observed meal, breakdown of the food within the gut being followed histologically by the methods listed.

The types of enzymes involved in digestion were investigated histochemically in similar series fixed at 4° C in 10% neutral formalin containing 3% sodium chloride. Sections were cut either directly on a freezing microtome or after rapid dehydration in cold acetone, clearing in xylol and infiltration *in vacuo* in paraffin wax melting point 45° C. Techniques used for enzyme identification included the Hausler (1958) and Hansson (1967) methods for carbonic anhydrase; the Hess and Pearse (1958) method for endopeptidase of the cathepsin-C type as used by Jennings (1962a, 1962b), Rosenbaum and Ditzion (1963) and Jennings and Mettrick (1968); the Burstone and Folk (1956) method for exopeptidases of the leucine aminopeptidase type; the Holt and Withers (1952) and Gomori (1952) methods for non-specific esterases; the Gomori (1952) and Abe, Kramer and Seligman (1964) methods for lipases; the Gomori (1952) and Burstone (1958) methods for acid phosphatase; and the Gomori (1939) method for alkaline phosphatase. Attempts were made to visualize carbohydrase activity using the methods of Pearse (1961) for β-glucuronidase (after Fishman and Baker, and Seligman, Tsou, Rutenburg and Cohen), and for α-glucosidase (after Rutenburg, Lang, Goldburg and Rutenburg).

Controls for these histochemical methods included the use of heat inactivated sections, media lacking specific substrates or containing specific activators or inhibitors, and the simultaneous processing of appropriate mammalian tissues.

The distribution of carbohydrate reserves was investigated in specimens fixed in 90% alcohol containing 1% picric acid, sections being stained by the Best's carmine or PAS methods for glycogen. Fat reserves were studied after fixation in Flemming's fluid or in frozen sections of formalin-fixed material stained in Oil Red O or Sudan Black B.

OBSERVATIONS

ANOPLA

Order: PALAEONEMERTINI

Cephalothrix bioculata and *C. linearis*

The two palaeonemertines studied show no significant differences in the structures and physiological processes concerned with nutrition and the following account is applicable to both species, unless stated otherwise at the appropriate point.

Structure of the gut and proboscis

The alimentary canal in both species is ciliated throughout its length and divisible morphologically into three distinct regions, the mouth and buccal cavity, the foregut, and the intestine (Fig. 1). The mouth is ventral and subterminal, 2–4 mm from the anterior end, and elliptical with its long axis running transversely to the main body axis. The deeply lobed lips and some slight folding of the buccal epithelium anteriorly allow the mouth to be distended during ingestion to a diameter equal to, or even slightly in excess of, the diameter of the body.

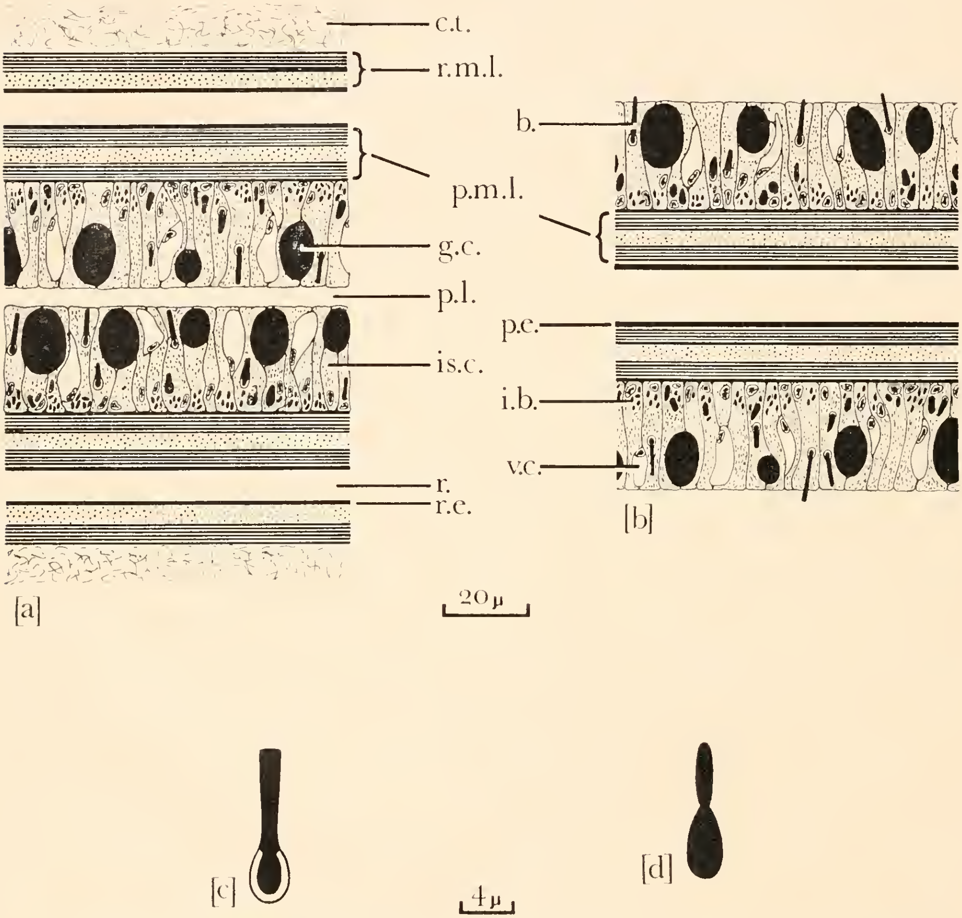


FIGURE 2(a). *Cephalothrix bioculata.* Schematic vertical longitudinal section through a portion of the proboscis and rhynchocoel as seen in the retracted position. (b) *Cephalothrix bioculata.* Schematic vertical longitudinal section through a part of the proboscis in the everted condition. b., barb; c.t., connective tissue; g.c., granular cell; i.b., immature barbs; is.c., interstitial cell; p.e., proboscis endothelium; p.l., proboscis lumen; p.m.l., proboscis muscular layers; r., rhynchocoel; r.e., rhynchocoel endothelium; r.m.l., rhynchocoel muscular layers; v.c., vacuolate cell. (c). Proboscis barb from *Cephalothrix bioculata.* (d). Proboscis barb from *Cephalothrix linearis.*

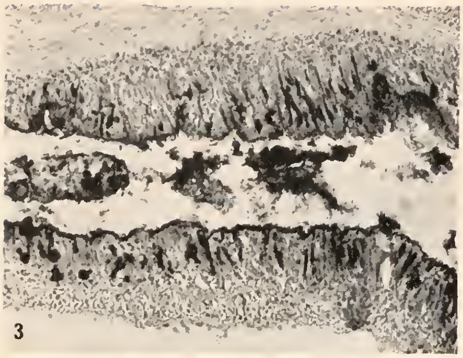
The buccal epithelium is made up of ciliated columnar cells 50–60 μ tall and 4–6 μ wide, with acidophilic and basophilic gland cells 18–20 μ by 5–7 μ interspersed amongst them. The majority of the basophils produce mucoid secretions, staining strongly with Alcian blue and PAS, which are discharged during ingestion presumably to facilitate passage of the food. The acidophils, in contrast, produce non-mucoid secretions and are extremely rich in carbonic anhydrase (Fig. 3), an enzyme normally associated in alimentary systems with production of hydrochloric acid. Cytoplasmic carbonic anhydrase can be demonstrated at all times, and when the acidophils discharge during ingestion of food the enzyme can also be found in the secretions poured on to the food as it passes onwards into the foregut.

The foregut occupies about one-fifteenth of the animal's length and consists of a simple unfolded ciliated tube. The epithelium is identical with that of the buccal cavity as regards both nature and frequency of the cell types present, but is reduced in height to 25–30 μ. The lumen is approximately 30 μ in diameter, narrowing to 15–18 μ at the junction with the intestine.

The intestine is the longest part of the gut and extends from its junction with the foregut direct to the anus at the posterior extremity. There is no cecum, but paired shallow lateral diverticula occur for most of the length up to a short pre-anal region which lacks these structures. The intestinal wall, or gastrodermis (Fig. 4), is composed of two cell types arranged as a single layer upon a thin basement membrane. The majority of the cells are columnar, 50–60 μ tall and 5–7 μ wide, sparsely ciliated and with prominent basal nuclei. Elongate pyriform gland cells, 35–40 μ by 7–8 μ and packed with acidophilic proteinaceous spheres 1.5 μ or less in diameter, occur proximally between the columnar cells. The spheres show a strong positive reaction to the Hess and Pearse method for endopeptidase of the cathepsin-C type (Fig. 4) and to the Holt and Withers, and Gomori, methods for non-specific esterase. Tracts of spheres often appear between the columnar cells, extending distally from the gland cells to the gut lumen. Smaller, more club-shaped forms of these gland cells also occur in the gastrodermis but in these the contained spheres are basophilic and react only weakly with the methods for enzyme visualization. Such cells never show tracts of discharged spheres leading from them towards the gut lumen and it would appear that they represent younger gland cells in the process of maturation.

Mature gland cells are abundant throughout most of the intestine but decrease in number posteriorly. Thus in the anterior region of the intestine in *C. bioculata,* for example, between 160 and 180 occur per 100 μ^2 of the gastrodermal surface, with a ratio of gland cells to columnar cells of about 1:4. Towards the anus, however, these values decrease to 30–35 and 1:20, respectively. In *C. linearis* the gland cells tend to be even more concentrated towards the anterior end of the intestine and here the ratio with the columnar cells is about 1:3, decreasing to 1:60 posteriorly.

The proboscis (Fig. 1) in both species in unarmed. It is a musculo-glandular tubular structure, which when retracted lies within the proboscis chamber or rhynchocoel and is confluent with the rhynchodaeum. This is a narrow tube opening to the exterior at the terminal proboscis pore. The proboscis can be everted through the rhynchodaeum by increased pressure within the fluid-filled rhynchocoel, and subsequently retracted by release of this pressure and contraction of the retractor muscle.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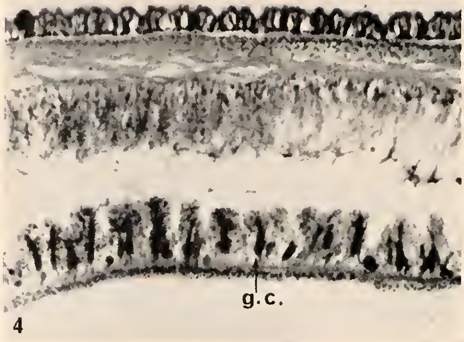
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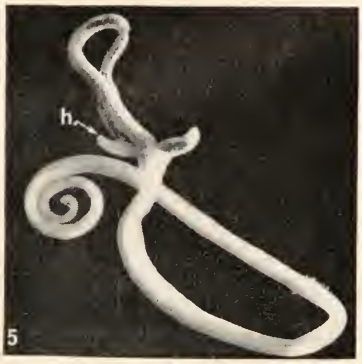
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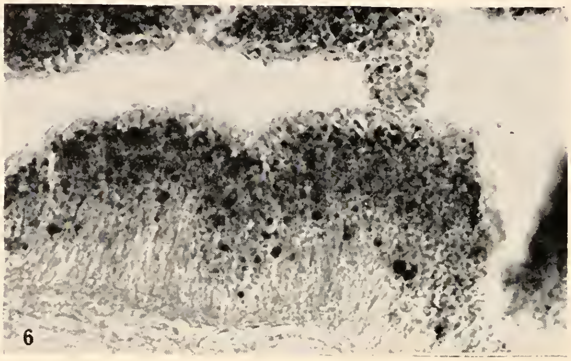
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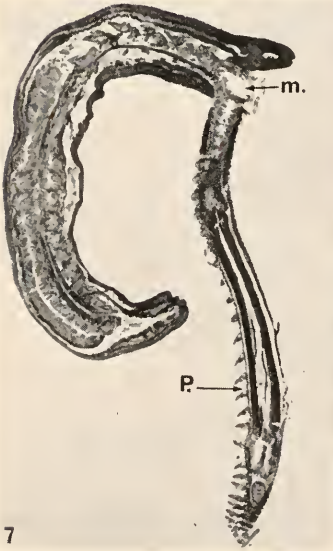
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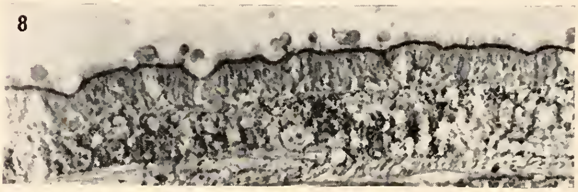
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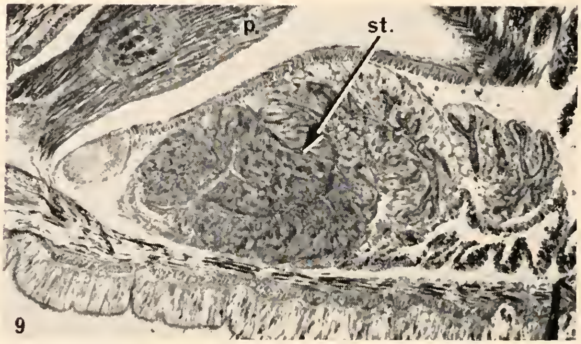
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FIGURES 3–9.

The rhynchocoel is lined by a thin endothelium overlying a thin band of muscle which in *C. bioculata* is composed of an inner layer of circular and an outer layer of longitudinal fibers. In *C. linearis* the outer muscular layer is oblique rather than longitudinal. The proboscis itself, in the retracted condition, is enclosed in a similar endothelium but possesses three muscular layers consisting of inner circular fibers surrounded on either side by longitudinal ones (Fig. 2a, b). The proboscis epithelium forms a smooth covering to the proboscis and is not thrown into papillae such as occur in the enoplan rhynchocoelans. In *C. bioculata* it is composed of three cell types, and four in *C. linearis*. In both species the commonest cell type is the interstitial cell, which is columnar, 25–30 μ tall and 3–8 μ in width. The distal cytoplasm contains up to ten acidophilic, proteinaceous and PAS positive rod-like structures, or barbs, very reminiscent of turbellarian rhabdites. When fully developed the barbs in *C. bioculata* (Fig. 2c) are 12 μ long and 1–1.5 μ wide with a bulbous proximal end which is inserted into a refractile cup 4–5 μ long and 2.5–3 μ wide. The point of insertion of the barb into the cup is in many instances distended into a slight collar-like structure. The barbs are arranged distally in tetrads and when the proboscis is everted they are protruded from the interstitial cells (Fig. 2b).

The barbs in *C. linearis* (Fig. 2d) are only 9–10 μ in length and lack a basal cup, but show a marked waist or constriction just over halfway along their length. They are not grouped into tetrads, but are occasionally paired.

The interstitial cells also contain proximally 8–10 simple rods which are considerably smaller than the barbs of the distal region but show the same staining reactions. They are believed to be the precursors of the fully differentiated barbs and presumably migrate distally to replace those protruded during proboscis eversion.

The other cell types present in the proboscis epithelium are oval vacuolated cells, 10–12 μ wide, and spherical forms which lack a nucleus when mature and

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FIGURE 3. *Cephalothrix linearis.* Longitudinal section through a portion of the buccal cavity (right) and foregut to show the acidophilic glands (black) which are rich in carbonic anhydrase. Hausler's method. Scale: 1 cm = 25 μ.

FIGURE 4. *Cephalothrix bioculata.* Longitudinal section of the gastrodermis. g.c., a gland cell showing a strong positive reaction for endopeptidase, lying between columnar cells. Hess and Pearse method. Scale: 1 cm = 50 μ.

FIGURE 5. *Cephalothrix linearis* photographed ingesting a newly captured *Tubifex*. The head (h.) is held upwards away from the mouth, which is distended into a funnel-shape and is grasping the *Tubifex* near its posterior end. Scale: 1 cm = 4 mm.

FIGURE 6. *Cephalothrix bioculata.* Longitudinal section of the gastrodermis prepared four hours after feeding and showing acid phosphatase activity (black) in the food vacuoles lying in the distal half of the columnar cells. The cytoplasm around the vacuoles shows a similar but less intense reaction. Burstone's azo dye method. Scale: 1 cm = 20 μ.

FIGURE 7. *Lineus sanguineus.* Longitudinal section of an individual fixed in Steinmann's fluid three hours after commencing ingestion of a *Phyllodoce* (P.). The nemertean's intestine is filled almost to the anus and the portion of the *Phyllodoce* still uningested is being separated off at the mouth (m.). Mallory. Scale: 1 cm = 0.5 mm.

FIGURE 8. *Lineus ruber.* Longitudinal section through the body wall, showing a narrow band (black) of non-specific esterase activity in the extreme distal region of the epidermis. Gomori's α-naphthyl acetate method. Scale: 1 cm = 50 μ.

FIGURE 9. *Amphiporus lactifloreus.* Longitudinal section through the anterior end showing a portion of the proboscis (p.) and the much-folded stomach (st.). Mallory. Scale: 1 cm = 50 μ.

possess very granular cytoplasm. These two cells form the supporting structure of the proboscis and are generally partially covered distally by the interstitial cells. *C. linearis* possesses a fourth cell type, irregular in shape, 15–20 μ in diameter and with acidophilic contents which are often aggregated into ovoid structures similar to the bases of the barbs. No indication of the function of these structures could be obtained.

The lumen within the retracted proboscis, lined by the epithelial layers, contains a coarsely granular lightly PAS positive secretion, and this is extruded when the proboscis is everted and forms a layer covering the epithelium and its protruding barbs. The source of this secretion is unknown, but it may originate from those cells of the proboscis epithelium which are not concerned in formation of the barbs.

The food and feeding mechanism

In the laboratory both species fed readily on living or dead oligochaetes. The freshwater *Tubifex* was convenient for use in observations on the feeding mechanism and was readily taken, but littoral forms such as *Clitellio arenarius* and littoral nematodes (*Pontonema sp.*) were also eaten. Freshly collected specimens generally showed little recognizable material amongst their gut contents but on occasion spherical structures, 1.5–2 μ in diameter and packed with small black granules, were found. These were very similar in size and staining properties to the chloragogenous cells present in most oligochaetes, including *C. arenarius,* and their presence in the intestine is taken as an indication that both species, under natural conditions, feed on littoral oligochaetes and similar organisms.

Both species lack cephalic furrows, which when present in other rhynchocoelans are believed to be the sites of chemoreception (Hyman, 1951), but despite this the feeding behavior indicates that prey is located chemotactically rather than as a response to mechanical disturbance of the water. Dead or damaged organisms are located and seized more quickly than living ones, but the introduction of oligochaetes in any condition elicits the same type of response. Individuals previously quiescent or merely moving slowly around their container become extremely restless when *Clitellio* or *Tubifex* are added, the head darts to and fro and often the body contracts and expands violently. The increased rate of movement soon brings the rhynchocoelan into contact with the food, but they do not "home" on to it as, for example, do many of the free-living flatworms. Introduction of inert, odorless objects does not elicit feeding behavior, and violently threshing prey are definitely avoided. Thus it would appear that food is detected chemotactically but the response is fairly generalized and depends entirely upon an increased rate of random movement.

When within range of living prey the proboscis is everted with explosive force and coils tightly around the prey's body. The prey is then drawn back towards the mouth by retraction of the proboscis, a process which may occupy up to thirty seconds, and during this time the prey becomes inert and apparently lifeless. Examination of the everted proboscis shows that in both species the barbs of the proboscis epithelium are protruded and penetrate the prey's integument. The barbs alone may be responsible for paralyzing the captured animal, but since a considerable quantity of secretions are always present in the lumen of the retracted proboscis it seems likely that these too are involved, and it is possible that the function of the barbs in this connection may be solely to puncture the prey and allow entry of proboscis secretions. An important secondary function may be to increase the grip of the proboscis on the struggling prey.

Inert foods, such as dead oligochaetes or other animal remains, do not cause proboscis eversion. Such materials, and killed prey brought to the mouth by the proboscis, are first "tested," the head being arched over them and moved slowly from side to side. The head is then bent back, the distended mouth is applied to the food and ingestion commences (Fig. 5). Ingestion is by suction, resulting from alternate contractions and expansions of the general body musculature, and is facilitated by copious secretions of mucus from the basophilic glands of the buccal cavity and foregut. Oligochaetes up to two-thirds the length of the nemertean were completely swallowed within three minutes and could be seen extending into the posterior intestine after a further three or four minutes.

Diameter, rather than length, of the food relative to the size of the mouth appears to be the critical factor determining whether or not ingestion is possible. Thus oligochaetes considerably longer than the nemertean will be swallowed, if of a suitable diameter, and when the foregut and intestine are completely filled the portion remaining protruding through the mouth is nipped off. This takes a little time and may well be caused by regurgitated digestive juices, supplemented by the constricting effect of the contracting mouth.

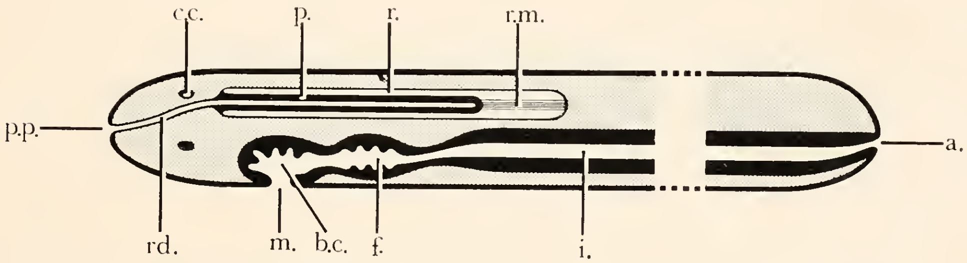


Figure 10. *Lineus sanguineus.* Schematic vertical longitudinal section to show the arrangement of the alimentary canal and proboscis, characteristic of the Heteronemertini. Abbreviations as in Figure 1.

The site and sequence of digestion

The acidophilic glands of the buccal cavity and foregut, which are rich in carbonic anhydrase (Fig. 3), discharge during ingestion. Foods stained with indicators show that the pH is considerably reduced as material passes on into the intestine and since carbonic anhydrase is known to be associated with acid production in most alimentary systems it is concluded that this is its role in the two species of *Cephalothrix* studied, the acid produced presumably facilitating subsequent proteolysis.

The intestinal acidophilic gland cells, whose contents react strongly with the methods for endopeptidase and non-specific esterase, discharge as food enters the intestine and their secretions retain their spherical form for a time. Within twenty minutes of feeding, however, the spheres have dissolved and previously enzymically inert boiled food begins to show peripheral endopeptidase activity as proteolysis commences. Eventually the entire contents of the intestine show this reaction, and become progressively more homogeneous, but no other enzymes could be demonstrated. Thus it would appear that extracellular digestion is entirely proteolytic and during this stage the pH of the gut contents is 5.5–6.0, as determined by application of indicators to samples withdrawn by means of a micropipette.

The duration of extracellular proteolysis depends directly upon the size of the meal. Phagocytosis of food particles commences within thirty minutes of feeding, as soon as proteolysis in the gut lumen begins to break up the food, and this continues until the columnar cells of the gastrodermis are loaded with food vacuoles. Material showing endopeptidase activity may still persist in the gut lumen at this stage if a large amount of food has been taken, but as intracellular digestion in the earlier food vacuoles is completed so new ones form distally in the columnar cells and this process continues until the lumen is emptied. This generally occurs within twelve hours of feeding but well before this time the lumen contents become quite homogeneous and nothing recognizable remains.

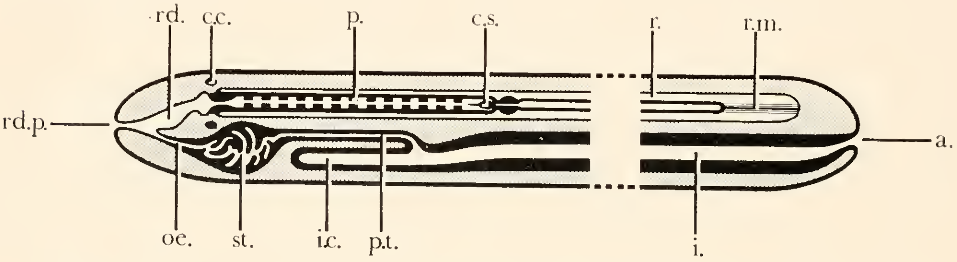


FIGURE 11. *Amphiporus lactifloreus.* Schematic vertical longitudinal section to show the arrangement of the alimentary canal and proboscis, characteristic of this type of Hoplonemertini. c.s., central stylet; i.c., intestinal cecum; oe. esophagus; p.t., pyloric tube; rd.p., rhynchodaeal pore; st., stomach. Other abbreviations as in Figure 1.

Contents of the food vacuoles continue to show endopeptidase activity for up to four hours after their formation but there is no evidence for the intracellular secretion of the enzyme or enzymes responsible. It is concluded, therefore, that the activity visualized in the vacuoles results from the enzymes originally acting extracellularly and phagocytosed with the digesting food. The vacuoles do, however, develop a strong reaction for acid phosphatase and a less intense but still easily visible reaction is found in the surrounding cytoplasm (Fig. 6). The role of this enzyme remains unknown, but it may be concerned with maintenance of the requisite acidic pH conditions in the vacuole, or perhaps with absorption of some early products of intracellular digestion.

Three to four hours after commencement of phagocytosis exopeptidases, as demonstrated by the Burstone and Folk method for aminopeptidases of the leucine aminopeptidase type, appear in the food vacuoles and rapidly increase in amount until after a further three hours, when virtually every vacuole in the gastrodermis shows an intense positive reaction. During this time vacuolar reactions for endopeptidase, non-specific esterase and acid phosphatase gradually decline and finally disappear. Eventually the aminopeptidase reaction similarly declines, as intracellular digestion progresses and the food vacuoles become reduced in size and number, and when digestion is completed and all vacuoles have disappeared exopeptidases cannot be visualized in any part of the gastrodermis.

Unlike the exopeptidase reaction, which can only be found in the gastrodermis during the later stages of intracellular digestion, a strong reaction for alkaline phosphatase can be obtained at all times in the distal regions of the columnar cells. Even in long-starved specimens the gastrodermis shows a clearly defined zone of activity distally, 2–4 μ in depth. During the formation of food vacuoles, however, this band of activity deepens and intensifies, and at the peak of exopeptidase activity the enzyme can be demonstrated in the cytoplasm throughout the cell and in virtually every food vacuole. On completion of intracellular digestion the activity fades and becomes confined once more to a thin distal band.

The distribution of alkaline phosphatase in the columnar cells, dependent as it is upon the particular stage of digestion of any one meal, suggests that this enzyme has two main functions. The first of these is concerned with normal cellular activities in the distal region of the cells, such as maintenance and renewal of the cilia, while the second is concerned either with production of the alkaline conditions necessary for exopeptidase activity or with secretion of these enzymes and the subsequent absorption of the products of digestion from the vacuoles.

Lipases and carbohydrases could not be demonstrated in the food vacuoles, but in the early stages of intracellular digestion mucus can be demonstrated in the distal regions of the columnar cells. This presumably represents a proportion of the mucus secreted by the buccal and foregut basophils, to facilitate ingestion, which has been phagocytosed along with food particles. The carbohydrate component of the mucus causes it to stain very strongly with both Alcian blue and PAS, but in later, older, vacuoles these reactions diminish very sharply and then disappear. Thus the presence of carbohydrases can be inferred, at least, in the absence of more direct evidence.

Food reserves

Fat forms the only significant food reserve in both *C. bioculata* and *C. linearis*. It occurs as droplets, varying in diameter from 1 to 5 μ, in the gastrodermal columnar cells and to a lesser extent in the parenchyma. The amount present at these sites decreases with starvation.

Very small amounts of glycogen were found in the gastrodermis of both species, occurring as minute particles scattered throughout the columnar cells.

Other sites of enzymic activity

In addition to the sites of enzymic activity in the alimentary system certain other sites within the body showed positive reactions to some of the enzyme visualization techniques.

In particular, the endothelial and gelatinous layers of the blood vascular system showed consistently in all specimens examined an intense positive reaction to the Burstone and Folk method for exopeptidases of the leucine aminopeptidase type. The reaction occurs completely independently of the nutritive state of the nemerteans, and of other factors such as the age or reproductive state. Full details of this phenomenon, which is common to all the rhynchocoelans examined in the present study, have been reported in a separate account (Gibson and Jennings, 1967).

Alkaline phosphatase activity was found in the parenchyma immediately adjoining the intestinal basement membrane, where it may well be concerned with transfer of nutrients from the columnar cells into the parenchyma, and in the endothelium and musculature of the rhynchocoel and proboscis. As in the case of the exopeptidases of the blood vascular system, this enzymic activity also is independent of the nutritive state or other discernable factors.

A weak reaction for non-specific esterase occurs at all times in the distal regions of the epidermis in both species, and slightly stronger reactions occur in the proboscis musculature and, occasionally, in the interstitial cells.

Order: HETERONEMERTINI

Lineus sanguineus and *L. ruber*

Structure of the gut and proboscis

The structure of the gut in *L. ruber* has been described in an earlier communication (Jennings, 1960) and consequently details need not be included here. *L. sanguineus* shows virtually no significant differences from *L. ruber*. Briefly, the gut in both species resembles that of *C. bioculata* and *C. linearis* in being divided into buccal cavity, foregut and intestine, but the walls of the buccal cavity and foregut are thicker and thrown up into prominent folds (Fig. 10). A further point of difference lies in the gland cells associated with the buccal cavity and the foregut, in that in the lineid species a considerable proportion of these occur in the underlying parenchyma and discharge through the gut wall into the lumen. These parenchymal gland cells include both basophilic and acidophilic types, the basophils producing mucoid secretions and staining strongly with Alcian blue and PAS. The acidophils are negative to Alcian Blue, stain only lightly with PAS, and their function remains unknown. Of the gland cells contained in the wall of the buccal cavity and foregut, the basophils similarly produce mucus and the acidophils are believed to produce acidic secretions used in killing the ingested prey, since they are rich in carbonic anhydrase.

The intestine, as in the two species of *Cephalothrix,* forms the longest part of the gut and, apart from short anterior and posterior portions, bears serially repeated lateral diverticula. These are up to 700 μ deep, compared with an intestinal width of only 600–650 μ and are frequently bifid distally. The anterior 10–12 pairs, and the posterior 10–16, are shallower and never bifurcated. In an average sized sexually mature adult *L. ruber* the diverticula give a three-fold increase to the internal surface area of the intestine, if the latter is considered as a central cylinder from which lateral pairs of smaller, closed cylinders emerge.

The intestinal wall, or gastrodermis, is identical in both the main region and the diverticula. As in the two *Cephalothrix* species, it is composed of ciliated columnar cells, 80–100 μ tall and 6–8 μ wide in *L. ruber* and 55–70 μ by 4–6 μ in *L. sanguineus,* and smaller pyriform acidophilic gland cells which lie proximally between the columnar cells. The gland cells contain spherical or oval acidophilic, proteinaceous globules showing strong positive reactions to the methods for endopeptidase and non-specific esterase. Tracts of secreted globules extend from the gland cells between the columnar cells up to the intestinal lumen. The gland cells are most numerous in the anterior intestine and decrease in frequency posteriorly, being completely absent from the short unpouched region immediately before the anus.

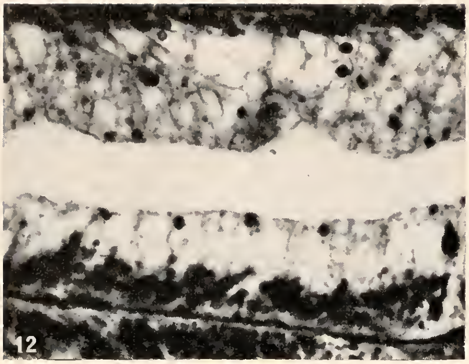
The proboscis (Fig. 10) in both species is unarmed and longer than in either *C. bioculata* or *C. linearis.* It lies coiled within the rhynchocoel, which runs dorsally above the intestine for most of the body length, and the principal difference between it and the cephalothricid type lies in the form of the epithelial rods. These are formed proximally in basophilic interstitial cells as tiny acidophilic rods, undifferentiated morphologically, which migrate to the distal border of the cells where they accumulate in batteries of fifty or more. They may be partially extruded in the retracted proboscis and in the fully everted one they are completely ejected from the formative cells. Their function would appear to be simply to increase the grip of the proboscis on the prey and not to facilitate entry into the latter of toxic secretions. Neither *L. sanguineus* nor *L. ruber* paralyze or kill the prey during capture, and the proboscis secretions other than the acidophilic rods serve merely to increase the effectiveness of the proboscis in gripping and holding a captured organism.

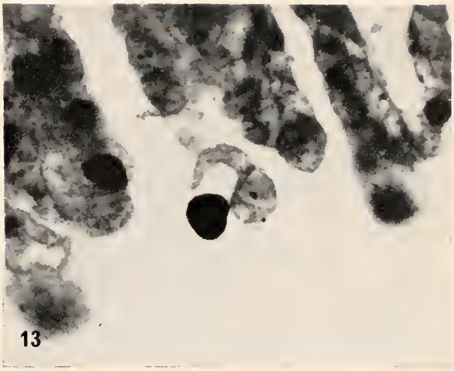
The food and feeding mechanism

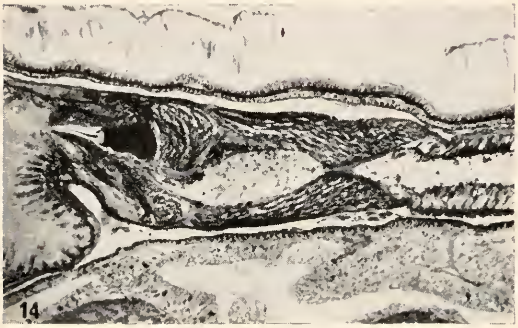
The two species of *Lineus* studied show interesting differences in food preferences. *L. ruber* feeds readily on living or dead oligochaetes, polychaetes, small crustaceans or any attractive organic material such as animal remains or clotted blood. In contrast, *L. sanguineus* will take only living food and feeds most readily on polychaetes such as *Phyllodoce maculata* and various syllids. Oligochaetes such as *Tubifex* or *Clitellio* are taken when the animal is starved, and occasionally other species of rhynchocoelans (*e.g., Amphiporus lactifloreus*) are also taken, but these are never captured and ingested as readily as polychaetes.

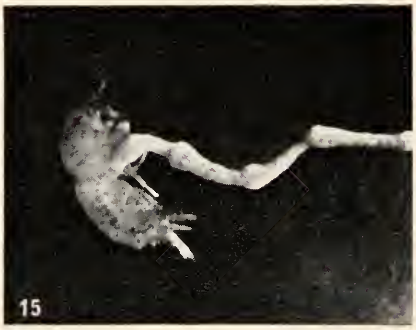
Both species detect prey chemotactically, and are able to locate damaged animals at distances of up to 8 cm. Intact living prey are detected visually within 2–3 cm of the head.

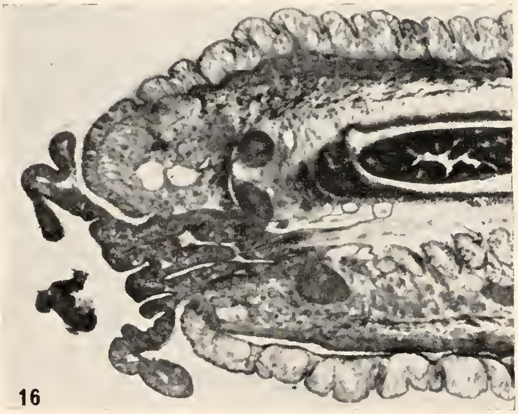
In both *L. sanguineus* and *L. ruber* living animals are captured by the proboscis. This is ejected when the prey is within range, coiled tightly around it and then retracted slowly to draw the struggling animal back to the head. The mouth is subterminal and the portion of the head anterior to it arches upwards and forwards to expose and dilate the mouth. The arched portion of the head may be used to grip the prey and hold it to the mouth, or it may be held clear. Small prey are swallowed within one minute of capture, head or tail first or in a U or J shape depending on the part of the body seized by the proboscis. Ingestion of larger organisms takes longer and *L. sanguineus* on one occasion took three hours to

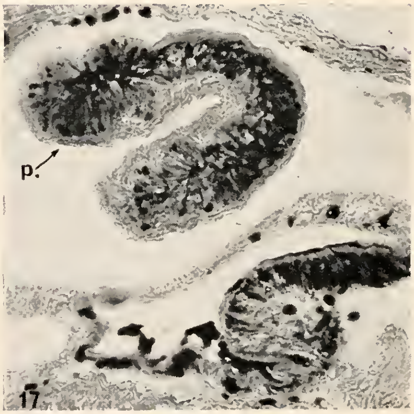












FIGURES 12–17.

FIGURE 12. *Amphiporus lactifloreus.* Longitudinal section of the gastrodermis in the posterior intestine of a starved specimen, showing columnar cells with basal acidophilic spheres which are the sites of endopeptidase activity. Mallory. Scale: 1 cm = 50 μ.

FIGURE 13. *Amphiporus lactifloreus.* Distal region of the gastrodermis of a specimen fixed 15 minutes after the start of a meal, showing the discharge of an endopeptidase-positive sphere into the gut lumen. Hess and Pearse method. Scale: 1 cm = 10 μ.

ingest part of a *Phyllodoce* which was considerably longer than itself. Ingestion continued until the entire intestine was filled (Fig. 7). This particular specimen was fixed and sectioned at this point, but in other similar instances the portion of the prey left protruding from the mouth was eventually nipped off and freed. Severance of the uningested portion appears to be effected partly by constriction of the mouth and partly by solution of the prey's body by regurgitated digestive juices, judging from the appearance of the end region of the discarded food.

The prey is ingested alive, thus confirming the absence of toxic proboscis secretions, but death usually occurs either as it passes through the foregut or very soon after entry into the intestine.

L. sanguineus refused all inert foods, but these were readily taken by *L. ruber*. As with *C. bioculata* and *C. linearis,* though, the proboscis was never everted and the nemertean simply extended the pre-oral portion of the head, dilated the mouth and engulfed the food.

The site and sequence of digestion

Animals ingested alive generally die as they pass through the foregut and experiments with indicators show that at this time the pH drops to around 5.0. Sections of newly fed individuals show that the acidophilic glands of the buccal cavity and foregut, which are rich in carbonic anhydrase, have discharged and it is concluded that the acidic secretions responsible for the drop in pH emanate from these cells. The mucus-producing basophils also discharge and considerable amounts of mucus can be found around the ingested food.

The endopeptidase-producing acidophils of the gastrodermis discharge as the food enters the intestine and previously enzymically inert materials soon show a positive endopeptidase reaction. From this point breakdown of the food follows virtually the same course as in the two *Cephalothrix* species, extracellular endopeptidase digestion being followed by phagocytosis and completion of proteolysis within the food vacuoles by exopeptidases demonstrable by the Burstone and Folk technique. The sequence of intracellular digestion, with the demonstration of exopeptidases, carbohydrases and lipases acting in concert, has been described in detail for *L. ruber* elsewhere (Jennings, 1962a) and *L. sanguineus* shows no significant differences. This earlier account, however, fails to report the occurrence of acid phosphatase in the gastrodermis and in the present work, using the

FIGURE 14. *Amphiporus lactifloreus.* Longitudinal section through the central region of the retracted proboscis, showing a portion of the anterior proboscis (left), the median bulbous region which contains the central stylet and its base and is expanded into a muscular bulb posteriorly, and a portion of the posterior proboscis (right). Mallory. Scale: 1 cm = 300 μ.

FIGURE 15. *Amphiporus lactifloreus.* Specimen photographed while feeding on a *Gammarus.* The head has been inserted within the crustacean, the stomach everted and contractions of the rhynchocoelan's general body musculature have begun to withdraw the prey's body contents. Scale: 1 cm = 2 mm.

FIGURE 16. *Amphiporus lactifloreus.* Longitudinal section through the anterior end of an individual fixed in Steinmann's fluid while feeding. The stomach is partially everted through the rhynchodaeal pore. Mallory. Scale: 1 cm = 100 μ.

FIGURE 17. *Prostoma rubrum.* Longitudinal section through the stomach (bottom right) and a part of the proboscis (p.) showing localization of carbonic anhydrase activity (black). Hausler's method. Scale: 1 cm = 75 μ.

Burstone azo-dye technique, this enzyme was found to be present in the cytoplasm of the columnar cells even during starvation. On formation of the food vacuoles the cytoplasmic activity shows a marked increase and large amounts of acid phosphatase appear in the food vacuoles. As with the two species of *Cephalothrix,* no definite role could be ascribed to this enzyme other than the suggestion that it may be concerned with maintenance of a low vacuolar pH or the absorption from the vacuoles of some earlier products of intracellular digestion.

Alkaline phosphatase activity occurs in the gastrodermis of both species, independently of the nutritive state, as a thin distal band. This increases in depth as the food vacuoles form and gradually increases in intensity in vacuoles and cytoplasm to a peak which coincides with the peak of exopeptidase activity. Thus in the early vacuoles both acid and alkaline phosphatases can be demonstrated but the relative strengths vary with time, the acid enzyme declining as the alkaline one increases.

Digestion is completed twelve to twenty-four hours after feeding, depending upon the amount of food taken, and indigestible residues consisting largely of inorganic fragments such as broken setae and occasional sand grains from the gut of the prey are ejected from the anus.

The food reserve

The principal reserve in both species consists of fat which is stored as droplets of up to 2.5 μ diameter in the gastrodermis and parenchyma. In mature females the ova also contain large amounts, in smaller droplets 0.2–0.5 μ in diameter.

Only small amounts of glycogen occur, in well fed individuals, as tiny granules scattered throughout the gastrodermis, parenchyma and musculature.

Other sites of enzymic activity

In *L. ruber* strong non-specific esterase activity, demonstrable by the Gomori α-naphthyl acetate method, occurs in a narrow distal band in the epidermis (Fig. 8) and in the cephalic furrows, foregut epithelium and the endothelium of the blood vascular system and protonephridial excretory system. Alkaline phosphatase and lipase were also found in the walls of the protonephridia, and acid phosphatase in small amounts in some of the epidermal gland cells. Exopeptidases occur in considerable amounts in the endothelial and gelatinous layers of the blood vascular system (Gibson and Jennings, 1967).

In *L. sanguineus,* exopeptidases are found at the sites showing non-specific esterase activity in *L. ruber,* namely the epidermis, cephalic furrows, foregut epithelium, and protonephridia. They occur also in the blood vascular system and in the distal regions of the interstitial cells of the proboscis epithelium. Alkaline phosphatase occurs in the protonephridia and, to a lesser extent, in the gonad endothelia and the ventral wall of the rhynchocoel. Acid phosphatase is found in the epidermal acidophilic glands and also in the material secreted by these glands on to the body surface.

The occurrence of these enzymes, at all the sites named in both species, is quite independent of the nutritive state of the animal.

ENOPLA

Order: HOPLONEMERTINI

Amphiporus lactifloreus

Structure of the gut and proboscis

A. lactifloreus, in common with many other hoplonemerteans, lacks separate external openings to the buccal cavity and rhynchocoel. A small oval aperture on the anterior tip of the body, the rhynchodaeal pore, opens into the rhynchodaeum from which the esophagus leads ventrally and the rhynchocoel dorsally (Fig. 11).

The rhynchodaeum epithelium consists of ciliated cuboidal cells 6–8 μ tall and lies over parenchymatous tissue containing oblique muscle fibers. It lacks gland cells, but mucus-producing cells occur in the underlying parenchyma and discharge through the epithelium around the rhynchodaeum.

The gut is divisible histologically into four parts, the esophagus, stomach, pyloric tube and intestine (Fig. 11). The intestine extends anteriorly beneath the pyloric tube as a blind cecum, and both intestine and cecum bear shallow, paired lateral multilobed diverticula.

The esophagus is a simple tube, 40–50 μ in diameter and about 1 mm long in an adult *A. lactifloreus.* It is lined by ciliated columnar cells 10–12 μ tall and 6–8 μ wide and lacks any glandular components. Posteriorly, the esophagus thickens and opens into the stomach, which has a thick, much folded and highly glandular wall (Fig. 9). This consists of densely ciliated acidophilic columnar cells, 50–70 μ by 5–8 μ, and large numbers of gland cells, 40–50 μ by 12–15 μ, which are loaded with finely granular intensely basophilic secretion. A proportion of the columnar cells appear to be secretory when fully developed, since acidophilic globules are often present in the distal regions and similar globules occur in the stomach lumen. The gland cells discharge either directly into the lumen or proximally between the columnar cells. In the latter case tracts of secretion extend from the gland cells up between the columnar cells and into the lumen.

Posteriorly the stomach wall becomes narrower, less folded and the proportion of gland cells diminishes as the stomach becomes continuous with the pyloric tube. This is non-glandular, lined by ciliated columnar cells, 25–35 μ by 3–5 μ, and somewhat folded in disposition. The tube narrows at its junction with the intestine.

The intestinal wall, or gastrodermis, is similar in structure to that of the other nemerteans studied, in that it consists of acidophilic gland cells packed with proteinaceous spheres and interspersed between ciliated columnar cells. The gland cells are most numerous in the anterior intestine, occurring in the ratio of one to every five columnar cells, but posteriorly the ratio drops to one in fifty.

Physiologically, however, both cell types differ markedly from their counterparts in *Cephalothrix* and *Lineus.* The contents of the gland cells show no reaction whatsoever to either the Hess and Pearse method for endopeptidase or the methods for non-specific esterase. They are discharged during the extracellular phase of digestion in the usual way, and are presumed to be enzymic, but their precise nature remains unknown. The columnar cells differ in that they contain up to 17–18 spherical acidophilic inclusions of variable diameter and these do show intense positive reactions for endopeptidase. The inclusions are not food vacuoles, as they appear in the basal regions of the cells in the absence of food (Fig. 12), increase in amount with time and are discharged distally as food enters the lumen (Fig. 13).

The proboscis in *A. lactifloreus* is armed with stylets, relatively long and lies coiled in the rhynchocoel for nearly the full length of the body. The rhyncocoel is lined by a thin endothelium, which overlies a muscular layer consisting of inner longitudinal and outer circular fibers.

The proboscis is differentiated into three regions, an anterior basophilic thick-walled tube, a short central bulbous portion housing the stylet apparatus, and a posterior acidophilic thin-walled region. The anterior tube, when retracted, consists of an endothelial layer enclosing four layers of muscle fibers which are, respectively, longitudinal, circular, longitudinal and circular in disposition. The inner epithelium, which forms the outer surface of the everted proboscis, is composed of columnar and gland cells and is thrown up into regularly arranged papillae. The papillae presumably increase the grip of the proboscis on the prey, aided perhaps by the secretions of the gland cells. These, however, are non-mucoid in staining reaction.

The median bulbous portion of the proboscis is in two parts (Fig. 11). The anterior region contains a central needle-shaped stylet, 140–145 μ long and 25–30 μ in diameter at its proximal end, which is borne on a sub-cylindrical stylet base 135–145 μ by 60 μ. The stylet is flanked by paired accessory pouches, each of which may contain up to six accessory stylets in various stages of formation. When complete each accessory stylet has the structure and dimensions of the central stylet. Lateral acidophilic gland cells discharge their secretions at the bases of the stylets and appear to be responsible for the formation of these structures.

The posterior part of the median bulbous portion of the proboscis is composed entirely of muscle fibers arranged around a narrow central lumen (Fig. 14). This opens anteriorly at the base of the central stylet and posteriorly is continuous with the cavity of the third, posterior region of the proboscis. This region is highly glandular, the wall containing both acidophilic and basophilic gland cells, some of which show a reaction for non-specific esterase, and the circular and longitudinal muscle layers are much reduced in thickness. The lumen contains a granular secretion derived from both types of gland cell, when the proboscis is in the retracted position, and it is believed that the secretion is ejected forwards into the wound caused by the central stylet when the proboscis is everted during the feeding process.

The food and feeding mechanism

During extensive laboratory tests the only food accepted by *A. lactifloreus* was the amphipod crustacean *Gammarus locusta*. All other amphipods, isopods and decapods offered failed to elicit a feeding response when presented alive, injured or dead, and the same result was obtained with a variety of oligochaetes, polychaetes and molluscs. No evidence was found to suggest detection of food from a distance and the nemertean appears to rely solely upon chance encounter with living *Gammarus* during random wanderings. When this occurs the proboscis is everted and coils tightly around the crustacean. The stylets penetrate the prey's cuticle and the posterior proboscis secretions pass through the wound into the body cavity.

During capture the *Gammarus* struggles violently but within forty seconds of proboscis eversion it becomes quiescent and the proboscis releases its grip and withdraws into the rhynchocoel. The proboscis is not used to pull the prey towards the head, as in the other species studied, and as it releases its grip so the nemertean moves forward until the head is in contact with the crustacean. The head and anterior region then make exploratory movements over and around the prey, apparently in a search for some weak spot in the integument such as occurs beneath the pleura and coxal plates. The head is then inserted into the prey and the stomach is everted through the rhynchodaeum (Figs. 15 and 16). If the head fails to gain entry the anterior portion of the proboscis is everted once more and applied to the *Gammarus*. It is held in one position for 1–2 minutes, with the end formed into a cup or sucker-like structure, and then withdrawn. The nemertean again attempts to insert its head, usually successfully, but if this is still not possible the procedure with the proboscis is repeated until the integument is breached. It is not known precisely how the proboscis achieves penetration, but it may be by mechanical action supplemented perhaps by some histolytic action of the secretions produced by the gland cells of the anterior proboscis.

The folding of the stomach and pyloric tube, in the resting condition, allows the stomach to be protruded well forwards through the rhynchodaeum and, with the nemertean's head actually within the prey, it is applied to the various organs and tissues of the body cavity. These become disorganized and partially broken down, suggesting the release of histolytic secretions from the glands of the stomach wall although these do not react to any of the histochemical methods employed for enzyme visualization. Portions of the *Gammarus* exoskeleton adjacent to the protruded stomach, however, develop a pink to red coloration, similar to that obtained when they are treated *in vitro* with 0.1 N hydrochloric, sulfuric or acetic acids. Thus it is concluded that a proportion, at least, of the stomach secretions are strongly acidic in nature, a conclusion supported by the intense basophilia shown by most of the stomach glands.

Material from the *Gammarus* appears in the intestine and cecum within three minutes of insertion of the nemertean's head and protrusion of the stomach. Ingestion results from strong, regular contractions of the circular muscles of the general body musculature (Fig. 15). Contractions commence in the region just posterior to the pyloric tube and pass down the body at the rate of 8–12 per minute. Ingestion occupies 30–40 minutes, with the head and stomach moving about within the *Gammarus*, and when completed usually little remains of the prey other than the empty exoskeleton. During the entire process the nemertean retains a firm hold on the substratum by pressing down the posterior third of the body and secreting considerable quantities of sticky mucus from the ventral surface of that region.

Dead specimens of *Gammarus* are ingested in the same manner as living ones, except that the initial proboscis eversion does not take place. Secondary eversion to penetrate the integument may still occur, however, if necessary.

The site and sequence of digestion

Material entering the intestine is at an acidic pH, and generally much disorganized, as a result of the secretions poured on to it from the stomach wall during ingestion. Carbonic anhydrase activity could not be demonstrated in the stomach or intestine, though, so acid formation in *A. lactifloreus* must involve some process distinct from that found in the other species studied.

The acidophilic gland cells of the gastrodermis discharge as food enters the intestine and cecum but, as noted earlier, it proved impossible to identify the enzymic component secreted. The columnar cells also discharge the spherical inclusions which they accumulate between meals (Fig. 13) and these show a strong reaction for endopeptidase both within the cells and after entry into the gut lumen. This reaction is taken up by the ingested food and as the number of spheres in the columnar cells decreases, there is a corresponding increase in the amount of enzyme activity demonstrable extracellularly.

Five to six hours after ingestion food vacuoles form in the columnar cells and contain material showing endopeptidase activity comparable in intensity to that seen in the gut lumen. The number of vacuoles increases with time, until all material from the lumen has been phagocytosed, but there is no increase in the intensity of the vacuolar endopeptidase activity, so that it can be concluded that there is no intracellular secretion of this enzyme into the vacuoles. Occasional spheres of endopeptidase remain within the columnar cells but these are clearly differentiated from the vacuoles.

Strong acid phosphatase activity occurs in and around the food vacuoles during this stage of intracellular digestion, which lasts for about thirty-six hours. The endopeptidase and acid phosphatase activity then declines and is replaced by exopeptidases and alkaline phosphatase at the same sites. These enzymes persist in the columnar cells until digestion is completed, when they disappear and cannot be demonstrated subsequently until the equivalent stage in the digestion of the next meal. This is the usual pattern for exopeptidase activity, as seen in the other nemertean species studied, but the absence of alkaline phosphatase from the gastrodermis at all times other than the terminal phase of digestion is quite exceptional.

Carbohydrases and lipases could not be demonstrated within the vacuoles, but their presence is inferred from the disappearance of carbohydrate and fatty components of the meal from the vacuoles.

The food reserves

As in the other species examined, fat forms an important food reserve in *A. lactifloreus*. It occurs principally in the columnar cells of the gastrodermis where very large amounts are stored in droplets of up to 4 μ diameter, although occasional globules of 10 μ diameter were found. The only other site of fat deposition is the ovarian endothelium and the mature ova, and it is absent from the parenchyma which is normally a lipid storage region in the nemerteans.

Extensive deposits of glycogen occur in the distal region of the gastrodermis, and lesser amounts in the parenchyma, musculature and gonads.

Other sites of enzymic activity

A narrow band of non-specific esterase activity 2–2.5 μ deep occurs distally in the epidermis and esophagus, and the epidermal mucus glands occasionally show acid phosphatase.

The other enzymes demonstrated in *A. lactifloreus* were exopeptidases associated with the blood vascular system, at the same sites as in *Cephalothrix* and *Lineus*.

Tetrastemma melanocephalum

The structure of the gut and proboscis

T. melanocephalum closely resembles *Amphiporus lactifloreus* in the histological structure of the gut and proboscis. Thus both organs open to the exterior via a common rhynchodaeum, the gut is divided into esophagus, stomach, pyloric tube and intestine, and the proboscis shows the same three regions and stylet apparatus as in *A. lactifloreus*. Such differences as do occur between the two species are only slight, and include a lesser degree of folding in the walls of the stomach and pyloric tube, the absence of paired diverticula from the intestinal ceca and a reduction in the number of acidophilic gland cells present in the gastrodermis. The contents of the gland cells, as in *A. lactifloreus*, show no reaction for either endopeptidase or non-specific esterase and their nature remains unknown.

The columnar cells of the gastrodermis contain varying amounts of acidophilic spheres, which show positive reactions for endopeptidase, and these appear to form in the basal portions of the cells and subsequently migrate distally to accumulate until food enters the intestine. The columnar cells also show food vacuoles, with contents undergoing intracellular digestion, and other vacuoles containing numbers of tiny black granules which are presumably indigestible residues from previous meals.

The food, feeding mechanism and site of digestion

T. melanocephalum proved to be a relatively rare species and only three individuals were available for the whole of this investigation. These three failed to feed upon any of the annelids, crustaceans or molluscs presented to them, and also refused dead animals and organic materials such as clotted blood and liver fragments. The gastrodermal columnar cells, however, consistently showed the aggregations of small black granules already mentioned and interpreted as representing residues of earlier meals. The granules were very similar in size and general appearance to the chloragogenous granules present in oligochaetes, and in the absence of further evidence it is suggested that annelids of this type form the principal component of the diet. It may well be that *T. melanocephalum* has a very rigid dietary preference, and that scarcity of the food organism, therefore, may be the factor controlling the occurrence of the species in any one habitat or at any given season.

The similarities in *T. melanocephalum* and *A. lactifloreus* as regards proboscis structure, stylet apparatus and gut structure all suggest that the feeding process is very similar in the two species. Certainly the folding of the walls in the stomach and pyloric tube in *T. melanocephalum* would appear to allow protrusion of the stomach through the rhynchodaeum, in the manner observed in *A. lactifloreus*.

Since specimens could not be induced to feed it was not possible to study in detail the site and sequence of digestion. However, as in the case of the feeding mechanism, sufficient evidence was available from the gut structure and the enzymes demonstrable to suggest that digestion follows much the same path as in *A. lactiflorcus.* Apart from the acidophilic endopeptidase spheres found in the columnar cells, which are believed to be responsible for extracellular proteolysis, the only other enzymes demonstrable in the gastrodermis were acid and alkaline phosphatase, associated with proximal vacuoles in the columnar cells. No exopeptidase activity was found, though, and there was no distal concentration of alkaline phosphatase in any part of the gut.

The food reserves and other sites of enzymic activity

No observations were made on the nature of the food reserves. Non-specific esterase activity was found distally in the epidermis, and exopeptidases were consistently present in the endothelium, gelatinous layer and plasma of the blood vascular system.

Prostoma rubrum

Structure of the gut and proboscis

The gut in *P. rubrum* is simpler than in the other two hoplonemerteans examined, in that it lacks both a pyloric tube and an intestinal cecum.

The terminal rhynchodaeal pore opens into the rhynchodaeum which is continuous anteriorly and dorsally with the rhynchocoel, and posteriorly with the esophagus. The latter is about 0.1 mm in length, slightly folded, and lined by cuboidal ciliated cells 6–8 μ tall. It opens directly, without any terminal constriction, into the stomach which is a bulbous, thick-walled organ some 200 μ long. The stomach wall is thicker dorsally, deeply folded and composed of three cell types. The commonest is columnar, 25–30 μ by 3–4 μ, with basophilic cytoplasm and densely ciliated distally. Pyriform gland cells, 10–15 μ by 4–5 μ and filled with spheres 0.5 μ or less in diameter, occur between the columnar cells and tracts of secreted spheres extend from them between the columnar cells into the gut lumen. The basophilic glands are PAS positive and the acidophilic ones negative, but the latter show an intense positive reaction for carbonic anhydrase (Fig. 17), indicating that they are concerned in production of acid. Both types are negative to Alcian blue.

Posteriorly the stomach wall becomes somewhat reduced in thickness and the gland cell content diminishes, but there is no clear demarcation into a pyloric tube as, for example, in *A. lactiflorcus.* A slight constriction marks the junction with the intestine.

The intestine bears shallow, paired diverticula laterally over most of its length, and its wall is virtually identical with that of the other hoplonemertean species. The columnar cells contain acidophilic endopeptidase spheres, apparently secreted proximally between meals and discharged distally on the entry of food into the intestine, and food vacuoles whose appearance and contents depend upon the time elapsed since the previous meal. The gland cells, occurring in the ratio of 1:30 with the columnar cells, are acidophilic and of the usual form and, as in *A. lactiflorcus* and *T. melanocephalum,* show no reaction for either endopeptidase or non-specific esterase.

The proboscis has the characteristic hoplonemertean form, being divided into an anterior basophilic region, a median bulbous portion bearing a central stylet and lateral accessory stylets, and a posterior acidophilic region. Histologically the proboscis is very similar to that of *A. lactifloreus,* but two important physiological differences occur. The acidophilic glands of the epithelium covering the anterior proboscis show strong carbonic anhydrase activity (Fig. 17), and a number of the acidophils of the posterior region give a reaction for endopeptidase. The secretion present in the posterior proboscis lumen, in the retracted condition, shows a similar endopeptidase reaction.

The food and feeding mechanism

P. rubrum fed in the laboratory on small living oligochaetes, and, on one occasion, a *Chironomus* larva. Small specimens of *Tubifex* were captured and ingested if the nemerteans were starved for seven days, but other oligochaetes such as *Aeolosoma* and *Stylaria* were taken much more readily. These species were abundant amongst the roots of floating water plants in the aquaria used for maintaining *P. rubrum* and the nemertean itself favored this type of habitat, rather than bottom debris or the leaves of bottom rooted plants. Thus the food in nature may well consist of oligochaetes of the *Aeolosoma* type, which occur in the selected microhabitat rather than those of the *Tubifex* type which are bottom dwellers in silt or mud.

The proboscis is used to capture the prey, being everted after a chance encounter with living oligochaetes. No evidence of either chemical or visual detection of food was found. The everted proboscis wraps tightly around the prey, which ceases movement almost instantaneously. Penetration of the integument by the stylet was not observed, but this and the injection of some paralyzing secretion can be safely inferred from the abrupt death of the prey once the proboscis has made contact. The secretions injected are presumed to be those of the posterior proboscis, which show an endopeptidase reaction, supplemented perhaps by acidic secretions from the anterior portion and originating in the glands rich in carbonic anhydrase.

The proboscis draws the inert prey back through the rhynchodaeal pore into the rhynchodaeum, where the food may be held for a short time while the proboscis is retracted fully into the rhynchocoel, and then passed rapidly through the stomach and into the intestine.

No evidence of stomach eversion was seen and the entire feeding mechanism resembled that seen in the anoplan species (*C. bioculata, C. linearis, L. sanguineus* and *L. ruber*), rather than that of *A. lactifloreus,* the only other enoplan species seen to feed in the laboratory. Dead oligochaetes, and living or dead crustaceans such as *Gammarus* and *Asellus,* did not evoke proboscis eversion and were not ingested.

The site and sequence of digestion

Since only a small number of specimens were available it proved impossible to study every stage of digestion. Sufficient information was acquired, however, to show that digestion follows the same course as in *A. lactifloreus* with an extracellular proteolytic phase, effected by endopeptidase secreted by the columnar cells, being followed by phagocytosis and completion of digestion intracellularly. The acidophilic gland cells of the gastrodermis discharge during the extracellular phase, but it proved impossible to identify their secretions or determine the part played by these.

The presence of carbonic anhydrase in some of the stomach glands indicates that their secretions are acidic in nature. Since the prey is killed before ingestion the secretions cannot have entirely the same function as those produced in the foregut of the anoplan species and they are not used in any form of extra-corporeal digestion as in *A. lactifloreus,* but they probably serve to denature the protein component of the food and to provide an acidic medium for the initial, extracellular proteolysis.

The food reserves

Fat occurs in the columnar cells of the gastrodermis, in droplets 3–3.5 μ in diameter and, to a lesser extent, in the gonads. Glycogen occurs at the same sites, with the larger deposits in this instance in the gonads.

Other sites of enzymic activity

Alkaline phosphatase occurs in the walls of the excretory ducts and acid phosphatase in a small proportion of the epidermal mucus glands.

The only other sites showing enzymic activities were the gelatinous and endothelial layers of the blood vascular system, where exopeptidases were consistently present as in all the other species of nemerteans examined.

Discussion

Of the seven species of rhynchocoelans investigated six are seen to be carnivorous and it is likely that the seventh, *Tetrastemma melanocephalum,* will also be found to take animal food, judging from the structure of the proboscis and alimentary canal and the occurrence of endopeptidase in the gastrodermis. The proboscis is used to capture active living prey, either with or without the supplementary use of stylets and an accompanying injection of paralyzing secretions, but inert or dead food if eaten at all is ingested directly and does not stimulate proboscis eversion. The digestive physiology, with strong emphasis on proteases and, in the majority of species, production of acid anteriorly in the gut, is clearly adapted to a predominantly animal diet. Similar findings on one or more of these aspects of feeding and digestion have been reported in the Anopla for various species of *Lineus* (McIntosh, 1873–74; Verrill, 1888–1892; Riches, 1893; Wilson, 1900; Piéron, 1914; Coe, 1943; Beklemishev, 1955; Tucker, 1959), *Carinoma, Parapolia, Tubulanus,* and *Zygeupolia* (Coe, 1943) and *Hubrechtella* (Hylbom, 1957). In the Enopla, too, similar findings have been reported for the hoplonemertine genera *Ototyphlonemertes* (Corrêa, 1948), *Geonemertes* (Hickman, 1963) and *Paranemertes* (Coe, 1901, 1943; Roe, 1967).

Thus it would appear that the situation found in the species investigated in the present study represents a pattern of nutrition characteristic of the Palaeonemertini and Heteronemertini in the Anopla, and of the Hoplonemertini in the Enopla.

The Enopla, however, includes a second order, the Bdellonemertini, which contains the single genus *Malacobdella* and this has been shown to be microphagous with a very high proportion of plant material in the diet (Gibson and Jennings, 1969). The basic feeding mechanism is completely different from that of other rhynchocoelans and has involved elaboration of the foregut into a pharynx which is used as a filtration mechanism. The digestive physiology is correspondingly modified and considerable emphasis is placed upon carbohydrases instead of proteases.

Thus in the Rhynchocoela there is a fairly intimate relationship between the diet, on the one hand, and the feeding mechanism, structure of the gut and digestive physiology, on the other; a situation very similar to that seen in the related Turbellaria (Jennings, 1957, 1968), monogenetic trematodes (Halton and Jennings, 1965) and digenetic trematodes (Halton, 1967).

Within the general pattern of nutrition in the carnivorous rhynchocoelans interspecific differences lie principally in the feeding mechanisms and one aspect of the digestive physiology, namely the site of production of the extracellularly acting endopeptidase.

With regard to feeding mechanisms, the main variation from the normal type is seen in *Amphiporus lactifloreus*. Here the head actually penetrates the captured *Gammarus* and the stomach, which is not present in the anoplan species, is everted to achieve first disintegration and then ingestion of the body contents. This type of feeding is well suited to crustacean prey which are enclosed in a tough protective exoskeleton and allows the rhynchocoelan to feed on *Gammarus* manifestly too large to be swallowed intact. Ingestion of large prey is within the capabilities of most of the species studied, but it is significant that in every case observed the ingested animal was either an oligochaete or polychaete annelid, with a relatively soft integument. *Lineus ruber* has been observed, on occasion, to ingest crustaceans intact but these were always very small relative to the rhynchocoelan and did not distend the body as did, for example, a large oligochaete. Presumably large crustaceans would prove resistant to digestion and *L. ruber* never attacked these. *A. lactifloreus*, however, kills *Gammarus* much larger in diameter than itself and is able to ingest the soft internal parts by virtue of its specialized feeding mechanism. In this respect, the stomach in *A. lactifloreus* is directly comparable with the protrusible plicate pharynx of triclad and polyclad Turbellaria, which is thrust into animals such as crustaceans, annelids, insect larvae and even colonial ascidians and used as a suction tube to withdraw body contents (Jennings, 1957, 1959).

Prostoma rubrum possesses the same type of alimentary canal as *A. lactifloreus*, with a well developed stomach, but does not prey on crustaceans. Oligochaetes are the chosen food organisms and the most effective way of dealing with these is apparently to ingest them intact. Eversion of the stomach does not occur and the entire feeding process resembles that seen in the Anopla.

Other differences in the feeding mechanisms of the species studied are relatively minor and are seen in the presence or absence of proboscis stylets. The Anopla lack stylets but in the two species of *Cephalothrix* the proboscis epithelium contains rhabdite-like barbs which apparently serve the same function as the more elaborate and larger stylets characteristic of the Enopla. Other, fluid, proboscis secretions are believed to be toxic, like those of the posterior proboscis in the Enopla, and the prey is killed during capture. In *Lineus sanguineus* and *L. ruber* barbs are present, but despite this the prey is ingested alive. Death occurs in the foregut, though, as the prey passes through and is exposed to the acidic secretions produced there.

Variation in the site of production of the extracellularly acting endopeptidase is the principal difference in the digestive physiology of the Anopla and Enopla. The gastrodermal gland cells produce this enzyme in the Anopla, but in the Enopla these glands, although virtually identical in structure and staining properties, show no reaction histochemically for any type of protease. In the Hoplonemertini (*A. lactifloreus, T. melanocephalum* and *P. rubrum*) production of endopeptidase for use in the intestinal lumen has apparently been taken over by the columnar cells but these still retain their phagocytic properties and digestion is completed within them in a sequence identical with that seen in the Anopla. The gland cells discharge their products into the gut lumen, but the part played by these remains unknown. In the other enoplan order, the Bdellonemertini, the diet contains a much larger proportion of carbohydrate and the gland cells are believed to produce amylases (Gibson and Jennings, 1969). Thus physiological modification of the gastrodermal gland cells, from the form seen in the Anopla, would appear to be characteristic of the Enopla, but it is difficult to see the functional significance of this in the Hoplonemertini which are entirely carnivorous.

The gastrodermal columnar cells in both the Anopla and Enopla are uniformly ciliated, but despite this they are also phagocytic. Conclusive proof that the contents of the food vacuoles result from true phagocytosis, rather than from concentration of absorbed soluble materials, was given by Jennings (1960) and Gibson and Jennings (1969), who showed that starch grains appear in the food vacuoles unaltered in both staining properties and optical activity.

This occurrence of phagocytosis in ciliated cells is of relatively rare occurrence in the animal kingdom and its significance in the Rhynchocoela remains uncertain. A study of the fine structure of the columnar cell has been undertaken to investigate this phenomenon and will form the basis of a subsequent account.

The food reserves in both Anopla and Enopla consist mainly of fat, a situation characteristic of most free-living animals, but no special modifications for storage or subsequent utilization occur.

Of the enzymes found at sites other than in the alimentary system the only types consistently present in all species are exopeptidases in the blood vascular system. A possible interpretation of this phenomenon, in terms of a peptide circulation analogous to the exocrinic-enteric circulation of amino acids described for the vertebrate intestine by Read (1950) has been fully discussed elsewhere (Gibson and Jennings, 1967).

Fisher and Cramer (1967) report that *Lineus ruber* can absorb amino acids and glucose across the epidermis, which possesses microvilli-like structures interspersed between the cilia. In view of these findings, it is possible that the nonspecific esterase found in the epidermis of *Cephalothrix bioculata, C. linearis, Lineus ruber, Amphiporus lactifloreus* and *Tetrastemma melanocephalum,* and the exopeptidase activity at the same site in *L. sanguineus,* may be concerned with extracorporeal digestion of simple proteins or polypeptides. The products of this digestion, if absorbed across the epidermis, would supplement the normal diet and their transfer about the body and subsequent metabolism may well be linked with the exopeptidase-peptide circulation complex of the blood vascular system.

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SUMMARY

1. A comparative study has been made of the food, feeding mechanisms, gut structure, digestive physiology and food reserves of representative species from three of the four orders of Rhynchocoela (Anopla: Palaeonemertini and Heteronemertini; Enopla: Hoplonemertini).

2. Polychaete or oligochaete annelids form the staple diet in the Anopla and one species of Enopla. *Amphiporus lactifloreus* (Enopla) is exceptional in that it feeds exclusively on the amphipod crustacean *Gammarus locusta*.

3. Living prey are captured by means of the proboscis, but inert or dead foods, when taken, are ingested directly without proboscis eversion.

4. In the Hoplonemertini the proboscis is armed with stylets and toxic secretions which kill the prey before ingestion, and a forerunner of this system is seen in the Palaeonemertini where minute epithelial barbs perforate the prey's integument to allow entry of paralyzing substances. In the Heteronemertini, though, the prey is swallowed alive and killed by acid secretions in the foregut.

5. In the Hoplonemertini the gut has an additional portion, the stomach, and in *Amphiporus lactifloreus* this is everted within the prey which is then ingested in fragments.

6. The basic digestive physiology is similar in both Anopla and Enopla, with extracellular acidic proteolysis being followed by phagocytosis and completion of digestion intracellularly by proteases, carbohydrases and lipases acting in concert. Intracellular digestion is in two phases, firstly acidic and then alkaline, and acid and alkaline phosphatases are associated with the appropriate phase.

7. The endopeptidase responsible for extracellular digestion is produced in the Anopla by gastrodermal gland cells but in the Enopla this function has been taken over by the columnar cells of the gastrodermis. Functional gland cells still occur in the Enopla but their role has not been determined.

8. The food reserves consist mainly of fat, stored in the gastrodermis in all species and, occasionally, in the parenchyma.

9. These findings are discussed in relation to previous work on nutrition in the remaining order of the Rhynchocoela (Enopla: Bdellonemertini).

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DEVELOPMENT OF ARTIFICIAL MEDIA FOR *ARTEMIA SALINA*[1]

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Much is known about the nutritional requirements of Insecta. This reflects progress in devising artificial media which meet the physical requirements of their varied types of feeding. Phytophagous, saprophagous, and parasitic insects have been fed on media brought to suitable consistency by agar or other solidifiers. Blood or plant-sap sucking insects present physical problems partly solved by feeding them on liquid media contained in membranes which can be pierced by the sucking organs. Little progress has been made with artificial media for filter-feeders. Yet such feeding is used by most aquatic invertebrates from sponges to arthropods, even by primitive chordates (*e.g.* sea squirts).

Filter-feeders are ostensibly phagotrophic but is phagotrophy obligate or facultative, *i.e.,* can the nutrient particles be replaced in part or totally by solutes? So far some mosquitos whose larvae are filter feeders were grown axenically; *Aedes aegypti* has been grown in chemically defined media, (Singh and Brown, 1957; Akov, 1962).

Artemia salina was selected for the present work because its eggs are commercially available, easy to purify and, because it tolerates a wide range of salinities, it might withstand high concentrations of organic and inorganic substances. A complex artificial medium capable of supporting growth from nauplii to adults had been devised (Provasoli and Shiraishi, 1959).

We describe here a simpler medium for *Artemia* and some nutritional problems presented by filter-feeders.

METHODS AND RESULTS

The bisexual tetraploid race of *A. salina* from the Great Salt Lake (Utah strain) was used throughout. Techniques for axenizing eggs, culturing nauplii, and testing sterility of inocula remained unchanged (Provasoli and Shiraishi, 1959).

Two methods were used to assess the adequacy of the media: *1*) *Serial transfer.* Nauplii (24–48 hr old) were inoculated into experimental media (5 ml) containing soluble (liquid phase) and insoluble nutrients (particulate phase). Surviving or developing organisms were transferred every 5 to 8 days into duplicate tubes (10 ml) until they stopped growing or died; *2*) *Drop-wise feeding.* The nauplii were inoculated into the liquid phase of the artificial media (10 ml) containing only the soluble inorganic and organic components and finely dispersible substances (solubilized fatty acids and sterols). The complete medium containing the particles (insoluble rice starch and heat-precipitated albumin and/or globulins), was added drop-wise to this liquid phase (1 ml aliquots) at inoculation, and every few days,

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i.e., as soon as the organisms cleared the particulates. When the organisms had become juveniles they were transferred to a 10-ml tube containing the complete medium.

To facilitate graphic comparisons of the experiments, growth was expressed as the number of molts per animal per volume of media (5 or 10 ml) obtained during the first 12–17 days of growth. The first 14 molts, culminating in an adult animal, are easily distinguished because of the changes in appearance. Adult artemias after the 14th molt continue to molt every few days but the morphological changes after each molt are subtle: gradual increase in the size of the claspers (2nd antennae of the male) and the ovisac. Each stage of the life cycle was identified by culturing many neonates to adults, each in a separate container, observing them twice daily, and identifying the instar from the number of exuviae produced and by examination of fixed "typical" animals (D'Agostino, 1965). Heath (1924), in basing the stages on frequencies of appearance in field collections, missed some instars. Precise designation can now be assigned to the working description of the stages recorded previously (Provasoli and Shiraishi, 1959): "metanauplii III small and big" = 3rd to 6th instar; "metanauplii IV "small" to "big" cover the span of the 7th to 11th instar; "juvenile" = 12th instar; "young" the 13th; and male and female, from the 14th instar on (Fig. 1).

An arbitrary method was devised to express growth numerically. It was difficult to measure growth because generally (in poorly balanced media) the 5 animals inoculated in one tube had different growth potentials. A similar variability was observed in the parthenogenetic strain of *A. salina* collected in Comacchio (Italy) by Dr. Barigozzi, even after several hundred parthenogenetic generations derived from the original single female. Parthenogenetic *Daphnia magna* showed the same phenomenon: 1–2 individuals out of 5 grow much faster. Typically, in 17 days growth in 10 ml of medium, 1 animal was at the 14th instar, 2 animals at the 12th, 1 at the 11th, and 1 at the 7th instar. Comparing nutritional variables thus posed a serious problem. We eventually scored individuals by assigning them an arbitrary value ("growth index") which approximates number of molts and size (Legend Fig. 1, A–I). The sum of these 5 numbers divided by 5 served as index of the total growth in a particular tube (in the above case 13 + 22 + 10 + 5 = 50:5 = 10.

This index served for numerical or graphical comparisons and proved reliable despite its crudeness.

Preparations

Starch particles Insoluble rice starch was ground in a colloidal mill; 100-mg portions were transferred in screwcap test tubes with some glass beads (1 mm diameter) then sterilized in a dry oven at 180° C for 2 hr, allowed to cool, and 10 ml of sterile distilled water were added aseptically (1 ml of suspension = 10 mg starch). This slurry was shaken and added aseptically to the experimental tubes.

Starch gel Insoluble rice starch was suspended in aliquots of medium (proportion 1 g in 40 ml of water), brought to a boil, then cooled. The gelled starch was added to the remainder of the medium and homogenized with a magnetic stirrer. The media were then dispensed and autoclaved. Starch not previously gelled if

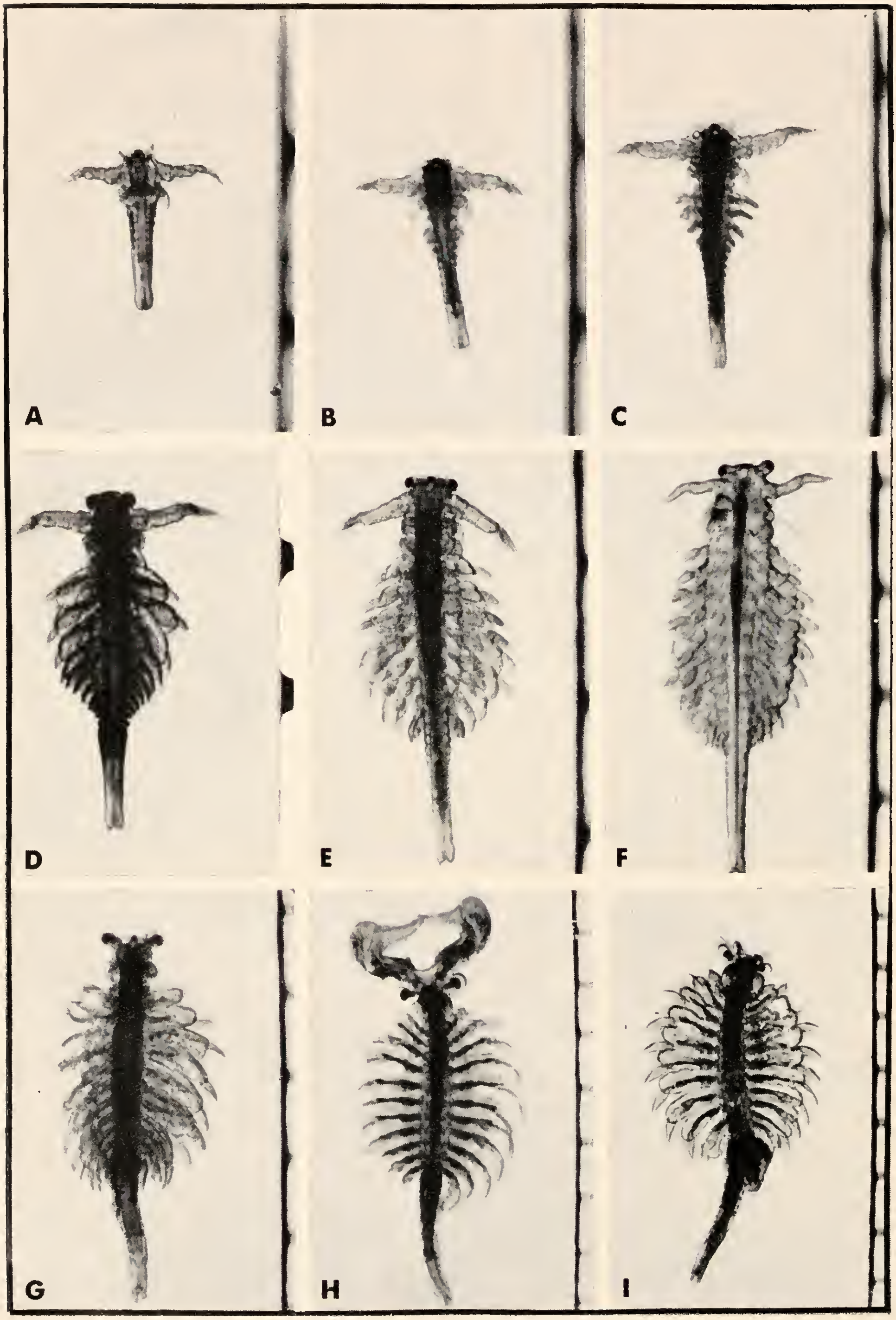

FIGURE 1, A–I.

suspended in media before autoclaving invariably formed aggregates and sometimes foamed out of the tubes during autoclaving.

Various sugars Concentrated stock solutions of soluble sugars were glass filter-sterilized and added aseptically to culture tubes.

Aseptic protein solutions Crystallin egg albumin, γ-globulin, and β-lactoglobulin were dissolved in either seawater or an equivalent salt solution and Seitz-filter sterilized. Such filtrates and commercial aseptic serum were added aseptically to culture tubes.

Protein particles One g of crystalline proteins (egg albumin, γ-globulin and/or β-lactoglobulin) was dissolved in 50 ml of seawater. The dissolved protein was transferred to a Virtis homogenizer; 10 g of glass beads were added (25 μ diameter; "Super Brite," Minnesota Mining and Manufacturing Co.). The solution was slowly brought to a boil and heated until protein precipitation was virtually complete. The precipitate was homogenized and autoclaved at 20 lb for 30 min. During autoclaving more precipitate formed; the coagulum was rehomogenized. Sufficient slightly buffered (bicarbonate) distilled water (pH 7–8) was added to resuspend the precipitate. The suspension was decanted into a stainless-steel sieve (0.038 mm pore size, Newark Wire Cloth Co., Newark, New Jersey) which retained the glass beads. The volume of the filtrate containing the fine suspended protein particles was readjusted to 50 ml by boiling off excess water. The final suspension contained 20 mg of protein per ml. This preparation was added to the final medium before autoclaving (15 lb, 20 min). The procedure was later modified to eliminate possible chemical contaminations: an artificial seawater was used; the glass-beads step was replaced with a more efficient high-speed 4-bladed homogenizer. This eliminated addition of the excess water needed for filtering off the glass beads and the subsequent boiling off of excess water (which may have caused unwanted hydrolysis of protein).

Liver Oxoid L-25 This water-soluble extract of beef liver (Colab Labs. Inc., Chicago Heights, Illinois) was used to supplement media with unidentified growth factors. It was added to media before autoclaving: during autoclaving a little brown precipitate forms.

Co-gels Mixtures of protein and starch were co-gelled to provide small particles containing both. This was done to favor growth of naupliar stages (especially of *Tigriopus*). Very fine particles are needed because of their small food-gathering parts. Since the gut of nauplii is small, the co-gel avoided excess feeding on either starch or proteins; selection of particles depending on size.

The proportions starch:albumin could be varied at will, but the proportion of solute to solvent had to be kept within narrow limits in order to obtain a firm gel

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FIGURE 1. Developmental stages of *A. salina*. A. Metanauplius III small = 3rd instar; 0.9–1 mm long; 24–48 hr. old; employed generally as inoculum. B. Metanauplius III big = 5, 6 instars; 1.3–1.5 mm long; growth index 4; terminal stage of growth if the medium is incomplete. C. Metanauplius IV small = 7, 8 instars; 1.6–1.9 mm long; growth index 5. D. Metanauplius IV medium = 9, 10th instars; 2.2–2.4 mm long; growth index 8. E. Metanauplius IV big = 11th instar; 2.4–2.6 mm long; growth index 10. F. juvenile = 12th instar; 2.7–4.0 mm long; growth index 11. G. young = 13th instar; 3.5–5 mm long; growth index 12. H. functional male = 19th instar; 6.5 mm long. A male ranges from 5 mm (14th instar, growth index 13) to 9 mm (25th instar). I. mature female = 15th instar; 6.5 mm; growth index 15. Females may reach 11 mm at 24th instar. (The scale is in millimeters.)

(~1:10–20 w/v). Typically, half a gram of insoluble rice starch was mixed well in a solution of crystalline egg albumin (0.1 g in 5 ml H_2O at pH 7.0), giving a ratio starch:albumin 5:1. The colloidal-suspension was slowly heated to a boil while continuously stirred. The gel was allowed to cool. Ten ml of water were added (pH 7.0), and the gel was homogenized, autoclaved at 20 lb for 30 min, cooled, and rehomogenized aseptically until finely suspended (milky consistency). It was transferred to sterile rubber-lined screwcap tubes. This preparation could neither be re-autoclaved nor stored in the refrigerator because the particle would re-aggregate; it remained stable for 15–30 days at room temperature.

The optimal ratio starch per protein seems to vary: for *Artemia* is 5–3:1 while *Daphnia* prefers 1:1 and tolerates well even higher ratios of proteins.

Cholesterol Twenty mg of cholesterol dissolved in 2 ml of 95% ethanol, were squirted with a small-bore needle into 20 ml of boiling water; a fine precipitate formed. The alcohol was evaporated in a water bath and the final volume was adjusted to 20 ml (1 ml suspension = 1 mg cholesterol). Aliquots of this suspension were added to media before sterilization. *Artemia* can withstand 1 ml/100 ethanol but *Daphnia* are sensitive to traces.

Cholesterol was sometimes used dissolved in warm propylene glycol and it was added as such to the media; 10–30 mg% of propylene glycol were non-toxic to *Artemia* or *Daphnia*.

Solubilized fat mixtures Several mixes proved useful and in most cases non-toxic for *Artemia*. A typical mixture, PFTC, was prepared as follows: *1*) *Phospholipid-fat preparation ("PF1")*. One g refined lecithin (Gliddex I, Central Supply Co., Chicago) was dissolved by mixing it thoroughly with 2 grams each of Tween 60 and 80, then 20 ml of propylene glycol (50% in water) were added. The whole was brought to 100 ml with H_2O; it should be clear. *2*)*Phospholipid-fat-cholesterol-taurocholate preparation ("PFTC")*. To 5 ml PF1 were added 3.5 ml of an alcoholic solution of cholesterol (10 mg/ml). After thorough mixing, the alcohol was boiled off, then 15 ml of a Na taurocholate solution (10 mg/ml) were added, brought to a boil and the volume adjusted to 50 ml with H_2O. This also should be a clear solution; 1 ml contained 10 mg propylene glycol, 2 mg each of Tween 60 and 80, 1 mg lecithin, 3 mg Na taurocholate, and 700 μg cholesterol. Used for *Artemia* at 1 ml% (V/V).

Refining the 1959 medium

The original medium was obtained empirically adding rich organic crudes to improve growth (medium "1959" Table I; Provasoli and Shiraishi, 1959). We assumed that particles were needed—starch had been selected, being relatively pure—and that cholesterol, nucleic acid components, and vitamins might be needed by the crustacea much as for fellow arthropods, the insects. Adults were finally obtained by the addition of serum, glutathione, "paramecium factor" (Lilly and Klosek, 1960) and higher vitamin levels; each of these additions improved growth but none dramatically.

The technique was to inoculate five 24–48 hr-old nauplii in 5 ml of the medium. Every 7 days they were transferred to new media (10 ml) whether or not they had consumed all the particles. On the average they became "metanauplii IV big"

TABLE I

Initial medium "1959" (% w,v/v)		Final medium "100" (% w,v/v)	
		NaCl	2.4 g
		$MgSO_4 \cdot 7H_2O$	0.6 g
Seawater	80 ml	$MgCl_2 \cdot H_2O$	0.4 g
H_2O	15 ml	$CaCl_2 \cdot 6H_2O$	0.22 g
Soil extract	5 ml	KCl	60 mg
		Metal mix PII[e]	3 ml
		Metal mix SII[f]	1 ml
		Fe (as Cl)	0.05 mg
K_2HPO_4	1 mg	Na_2 glycerophosphate	50 mg
		Adenylic acid	60 mg
Alk. hydr. RNA[a]	40 mg	Guanylic acid	2.5 mg
Acid hydr. DNA[b]	10 mg	Cytidylic acid	5.0 mg
		Uridylic acid	5.0 mg
		Thymidine	2.5 mg
Liver infusion	100 mg		
Horse serum	5 ml	Egg albumin **P**	10–20 mg
Trypticase BBL	320 mg	L-Threonine*	20 mg
Paramecium factor[c]	5 mg	L-Histidine*	20 mg
Yeast autol. Difco	20 mg	L-Phenylalanine*	10 mg
Glycine	10 mg	L-Serine*	40 mg
L-Glutamic ac.	50 mg	L-Glutamic ac.*	100 mg
DL-Alanine	10 mg		
		Glucose	300 mg
Sucrose	300 mg	Sucrose	300 mg
Rice starch **P**	500 mg	Rice starch **P**	100 mg
Cholesterol **P**	200 μg	Cholesterol **P**	800 μg
Glutathione	30 mg		
Ascorbic ac.	3 mg	Vitamin mix A.VIII[g]	2 ml
Vitamin mix A.II[d]	1 ml	Glycylglycine	100 mg
pH = 7.4		pH = 7.4	

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[a] Yeast RNA brought to pH 9.0 with NaOH and steamed for 1 hour.

[b] Herring sperm DNA brought to pH 1.5–2.5 with H_2SO_4 and steamed for 2 hours.

[c] Kindly supplied by Dr. Daniel M. Lilly; see Lilly and Klosek, 1961.

[d] 1 ml Vitamin mix AII contains: thiamine HCl 0.1 mg; biotin 5 μg; folic acid 70 μg; nicotinic acid 0.5 mg; choline 5 mg; Ca pantothenate 0.7 mg; pyridoxine HCl 80 μg; carnitine 0.2 mg; riboflavin 1 μg.

[e] 1 ml metals PII contains: Na_2 EDTA 1 mg; Fe (as Cl) 0.01 mg; B (as H_3BO_3) 0.2 mg; Mn (as Cl) 0.04 mg; Zn (as Cl) 5 μg; Co (as Cl) 1 μg.

[f] 1 ml metals SII contains: Br (as Na) 1 mg; Sr (as Cl) 0.2 mg; Rb (as Cl) 0.02 mg; Li (as Cl) 0.02 mg; Mo (as Na salt) 0.05 mg; I (as K) 1 μg.

[g] 1 ml of Vitamin mix A.VIII contains: thiamine HCl 1.2 mg; nicotinic acid 2.4 mg; Ca pantothenate 4 mg; pyridoxine HCl 100 μg; riboflavin 300 μg; folic acid 0.7 mg; biotin 60 μg; putrescine 200 μg.

P = particles.

* = components of amino acid mixture AA 1A (3.3 ml, see Table 3).

(11th instar) in 12 days, "young" in 15, "young adults" (14th instar) in 20, and full-grown adults in 37 days.

The liquid part of this medium (*i.e.*, without starch) supported no growth beyond the 4th instar (*i.e.*, full resorption of yolk and beginning feeding) indicating that the ingestion of liquids might not suffice to support growth; it was assumed that the starch particles either increased inbibition or might have absorbed the soluble nutrients, making them available by phagotrophy.

Replacement of complex substances Table II gives the compositions of 3 typical basic media which were developed during the stepwise replacement of the crude organic substances with chemically defined or simpler components. Replacement of liver infusion, horse serum, and yeast autolysate was difficult because these preparations contain a great array of nutrients: proteins, amino acids, polysaccharides, vitamins, fats, sterols, and emulsifiers. It was easy to replace Trypticase with an amino-acid mixture. Each time a crude was eliminated the components of the medium had to be rebalanced and often new factors had to be added.

The first successful elimination of serum, yeast autolysate, and liver extract (Table II, medium 31) was by replacing them with *a*) γ-globulin + β lactoglobulin + albumin; *b*) a more complete, far more concentrated mixture of B-vitamins; and *c*) by raising the cholesterol to 300 μg%. However specimens of *Artemia* in medium 31 grew more slowly than in medium 1959 and they generally failed to become sexually mature.

Of all the substances eliminated from the old medium, the liver infusion seemed richest in substances supporting sexual differentiation and oogenesis. However numerous complete amino-acid mixtures and vitamin mixtures failed to stimulate oogenesis even though the amino acids allowed longer survival of adults.

Medium 39 summarizes the efforts at simplification (Table II). Seawater was replaced with an artificial salt solution like the one employed for marine algae (Provasoli, McLaughlin and Droop, 1957). In doing so we found that Tris [(hydroxymethyl)aminomethane] inhibited *Artemia* while glycylglycine was well tolerated at pH-buffering concentrations (*Daphnia* behaved similarly). Glutathione and the "paramecium factor" proved superfluous. In an attempt to eliminate the tedious preparation of filter-sterilized protein solutions, and its risky aseptic addition to all culture tubes, precipitation by heat was tried. Unexpectedly, growth improved. But the addition of more particles seemed inhibitory, consequently the starch was reduced to 0.5% and the total proteins to 0.1%. A new, more concentrated vitamin mixture was used.

The failure to replace liver with amino acids and vitamin mixtures resulted in trying components of liver hitherto disregarded. Crude bile salts at 5–10 mg% replaced 0.05–0.1% of liver infusion. In turn, 3 mg% of Na taurocholate + higher cholesterol (500 μg%) replaced the bile salts. Since bile salts and taurocholate are potent emulsifiers, it was logical to suppose that fat-soluble factors might be involved and that cholesterol might be more effective in emulsion form (it was previously added as an ethanolic solution; after boiling off the alcohol it became a fine precipitate). Several emulsions were tried. Of these, PFTC (see "preparations") proved effective bringing in Tween 60 and 80, [thus providing oleic, palmitic, stearic, and traces of linoleic, and linolenic acids (Shorb and Lund, 1959)], taurocholate, cholesterol, and refined lecithin.

TABLE II

Medium 31 (% w,v/v)		Medium 39 (% w,v/v)		Medium 91 (%. w,v/v)	
		NaCl	3 g	NaCl	2.4 g
		$MgSO_4 \cdot 7H_2O$	0.7 g	$MgSO_4 \cdot 7H_2O$	0.6 g
H_2O	10 ml	$MgCl_2 \cdot 6H_2O$	0.4 g	$MgCl_2 \cdot 6H_2O$	0.4 g
Seawater	85 ml	KCl	0.08 g	KCl	0.06 g
Soil extract	5 ml	$CaCl_2 \cdot 6H_2O$	0.22 g	$CaCl_2 \cdot 6H_2O$	0.22 g
NaCl	0.5 g	Metal mix PII	3 ml	Metal mix PII	3 ml
Metal Mix PII	3 ml	Metal mix S7[a]	0.5 ml	Metal mix S7[a]	0.5 ml
Fe (as Cl)	50 µg	Fe (as Cl)	50 µg	Fe (as Cl)	0.05 mg
		Glycylglycine	0.1 g	Glycylglycine	0.1 g
K_2H PO	10 mg	Na_2glycero $PO_4 \cdot 5H_2O$	10 mg	Na_2glycero $PO_4 \cdot 5H_3O$	50 mg
Alk. hydr. RNA	30 mg	Alk. hydr. RNA	40 mg	Alk. hydr. RNA	80 mg
Acid hydr. DNA	20 mg	Acid hydr. DNA	10 mg	Ac. hydr. DNA	20 mg
Egg albumin	60 mg	Egg albumin **P**	60 mg		
γ-Globulin	60 mg	γ-Globulin **P**	20 mg		
β-Lactoglobulin	80 mg	β-Lactoglobulin **P**	20 mg	Egg Albumin **P**	20 mg
Glycine	10 mg	Aminoacids AAP_1[b]	6 ml	Amino acids AA18L[b]	6 ml
L-Glutamic acid	30 mg	Amino acids AAP_2[b]	1 ml		
DL-Alanine	10 mg				
Amino acids AAP_1[b]	5 ml				
Rice starch **P**	0.7 g	Rice Starch **P**	0.5 g	Rice Starch **P**	0.1 g
Glucose	0.4 g	Sucrose	0.3 g	Sucrose	0.3 g
		Glucose	0.3 g	Glucose	0.3 g
		Propylene glycol*	10 mg		
		Tween 60 + 80 (1:1)*	4 mg		
Cholesterol **P**	0.3 mg	Lecithin*	1 mg	Cholesterol **P**	0.8 mg
		Na taurocholate*	3 mg		
		Cholesterol*	0.7 mg		
Glutathione	30 mg				
		Vitamin mix A.V[d]	1 ml	Vitamin mix A.VIII[e]	2 ml
Vitamin mix A.III[c]	1 ml				
		pH = 7.4			

* components of PFTC mixture, see "preparations."

P = particles.

[a] 1 ml metal mix S7 contains: Br (as Na) 6 mg; B (as H_3BO_3) 0.4 mg; Sr (as Cl) 0.7 mg; Rb (as Cl) 0.01 mg; Li (as Cl) 0.02 mg; I (as K) 5 µg; Mo (as Na salt) 5 µg; F (as Na) 0.1 mg.

[b] see Table III.

[c] 1 ml vitamin mix A.III contains: thiamine HCl 0.17 mg; nicotinic acid 0.5 mg; Ca pantothenate 1 mg; pyridoxine HCl 50 µg; riboflavin 3.5 µg; folic acid 0.2 mg; biotin 15 µg; putrescine 45 µg; inositol 1 mg; choline citrate 1.5 mg; PABA 50 µg; vit. B_{12} 1 µg; carnitine 0.2 mg; orotic acid 0.3 mg; thymine 80 µg.

[d] 1 ml vitamin mix A.V. contains: thiamine HCl 0.3 mg; nicotinic acid 0.7 mg; Ca pantothenate 1.5 mg; pyridoxine HCl 25 µg; riboflavin 10 µg; folic acid 0.25 mg; biotin 20 µg; putrescine 50 µg; inositol 0.3 mg; choline citrate 0.5 mg; PAPA 50 µg; vit. B_{12} 1 µg; folinic acid 10 µg; carnitine 0.2 mg.

[e] see footnote [g], Table I.

Medium 39 was better than medium 31 in all respects and was inferior to the undefined medium "1959" only in that only rarely females produced eggs. *Artemia* in medium 39 developed faster, grew larger, and lived longer than in any other simplified medium and equaled medium 1959. The consumption of media per animal was also considerably reduced. Although sexual differentiation was now normal, oogenesis was induced only by adding 0.05–0.1% liver infusion: liver was supplying further factors.

In Medium 39, and in previous media as well, there was considerable mortality of the inoculum. Often >50% of the nauplii died within 24–48 hr of their transfer from the hatching medium (STP, Provasoli and Shiraishi, 1959) into experimental media. Such mortality necessitated re-inoculation of experimental tubes, increasing the risk of bacterial contamination and the concomitant undesirable addition of more STP, *i.e.,* additional unknown substances. Various factors were found responsible for the high mortality. The Utah *Artemia* nauplii hatch from the wintering eggs with enough yolk to complete 3 molts; therefore exogenous nutrients are not needed during the first 36 hr.

The non-feeding 0–18 hr old nauplii died because the finely suspended particles adhered to the bristles of the second antennae thus impeding swimming. Old nauplii (24–48 hr) were more tolerant but not completely safe from the effects of particles.

Older nauplii (48–72 hr or older) grown in fluid STP were starving and unable to recover especially in suboptimal media.

To inoculate the more resistant older nauplii it was necessary to avoid starvation after they exhausted their yolk. Addition of 4–6 drops of complete medium 39 to 10 ml of STP after the nauplii were 24 hr old was successful: larvae grew rapidly and mortality was negligible (<10%); additional drops of medium 39 were added afterwards as the particles were consumed.

The experimental technique was then changed: *a*) the 24-hr nauplii were inoculated directly into each experimental tube containing only the liquid part of the medium (10 ml), then the particulate medium containing the same nutritional variable was added dropwise (1 ml at a time, as the particles were consumed); nutritional carryover was thus avoided; *b*) 5 nauplii were still inoculated in each tube but growth was scored at 12 or 17 days because by then the results were clear: adults (>14 instar) were often obtained in 15–17 days as opposed to 33 days with the serial transfer system. Important results were checked periodically with replicate experiments. Several requirements were determined with this technique. The vitamin needs were determined by the usual subtraction tests, resulting in vitamin VIII mix (Provasoli and D'Agostino, 1962). It was also found that: higher concentrations of nucleic acid components were needed for faster growth; that cholesterol at a much higher level (0.8 mg%) was the only ingredient in the PFTC mixture needed (taurocholate and bile salts supplied cholesterol), and that albumin was the best protein thus eliminating the globulins.

The dropwise feeding technique, although useful, was not a practical solution as a growth medium: it was laborious and required stringent aseptic techniques to avoid infections during the repeated opening of the tubes. It was then desirable to return to a more conventional procedure. We knew that the total quantity of particles had to be reduced to avoid nauplii mortality but what was the optimal amount to maintain a high growth rate? This led to inquiries on the role of particles, (see below) and to a practical solution: use of 0.1% starch + 0.03% or 0.02% albumin.

These advances were embodied in medium 91 which served for studies on replacing the particulate starch and precipitated protein with soluble carbon and nitrogen sources and for the determination of the purine-pyrimidine requirements. In Medium 100 (Table I) all nutrients aside from starch and albumin are defined. Mortality in this medium was low (<10%) and adults (>15 instar) were obtained in 23 days after 2 transfer to 10 ml of new medium. Medium 100 still lacks the factors, supplied by liver infusion, for oogenesis. Without the addition of liver extract females carried eggs at 39 days but died at 45 days without depositing the eggs. With the addition of 75 mg% of liver extract females carrying eggs were obtained after 21 days and deposition after 25.

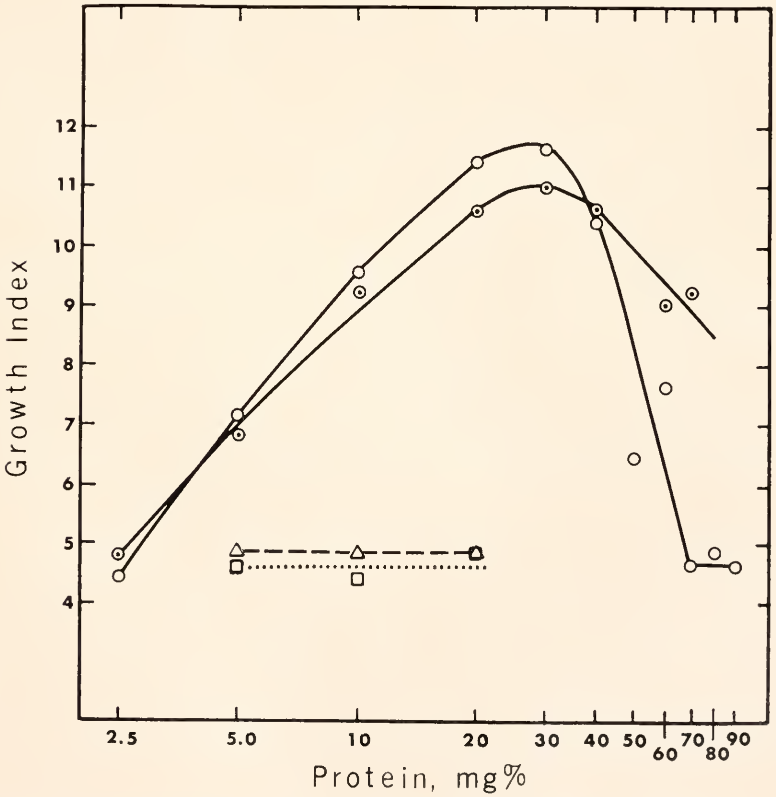

FIGURE 2. Relationships between constant particulate starch (100 mg%) and varying quantities of precipitated proteins. ○ = precipitated proteins (albumin 6 p + γ globulin 2 p + β lactoglobulin 2 p); exper. 152; 10 ml medium 91 (no amino acids); 5 animals; 12 days growth. △ = γ-globulin; exper. 152, same conditions. □ = β-lactoglobulin; exper. 152, same conditions. ⊙ = egg albumin; exper. 180; 10 ml medium 100 (no amino acids); 5 animals; 12 days growth. *Growth index,* see Material and Methods.

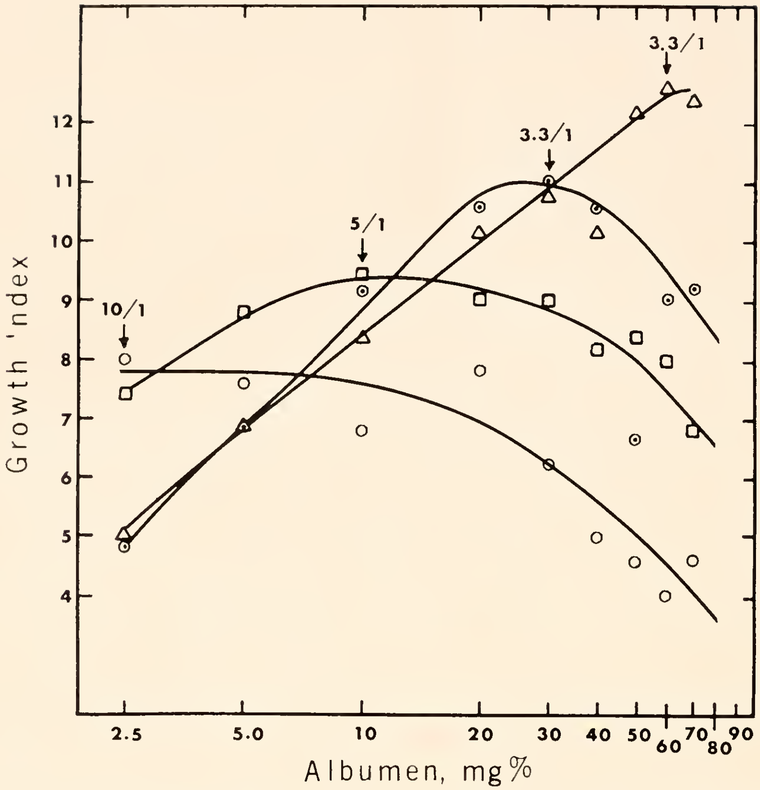

FIGURE 3. Relationship between 4 levels of particulate starch and varying quantities of precipitated albumin. △ = starch constant at 200 mg%. ⊙ = starch constant at 100 mg%. □ = starch constant at 50 mg%. ○ = starch constant at 25 mg%. Conditions: Exper. 180; 10 ml medium 100 (no amino acids); 5 animals; 12 days growth. ↓ = numbers stand for optimal starch/albumin ratio.

Phagotrophy and osmotrophy

Studies on rats and chickens have shown that for efficient diets the components must be balanced. Since in medium 39 the major components, carbohydrates and protein were particulate, it became necessary to vary their ratios and total concentrations to detect possible competition between them and to establish optimal conditions.

Starch:protein ratios To determine optimal ratios, the insoluble starch was kept at 0.1% while varying the protein from 1 to 100 mg%, hence the total concentrations in particles varied from 0.101–0.2%. Fig. 2 shows a typical run; as noted earlier, the growth index was the average growth per animal based on the growth achieved by 5 nauplii in 10 ml medium (see "materials and methods"). The nutritional value of the components of the protein mixture was analyzed. Albumin was the essential component even though the protein mix elicited slightly higher growth. Both became inhibitory above 0.04% and albumin was less inhibitory than the protein mixture. The optimal starch:albumin ratios were between 5:1 and 2.5:1. Obviously development and growth in the suboptimal range (1–10 mg%) depended chiefly on the quantity of protein.

In another series of experiments starch was supplied at 4 levels,—0.025, 0.05, 0.1, and 0.2%—and albumin varied from 1 to 100 mg% (Fig. 3).

As the level of starch increased more albumin was needed for optimal growth and at the lower levels of starch, albumin became inhibitory confirming the need for a ratio starch:albumin somewhere between 5:1 and 3.3:1.

Evidently both ratios and total particles affected growth making it necessary to keep total particles constant while varying the starch protein ratios from 1:1 to 30:1. This was done by inoculating 5 individuals in 5 ml of liquid medium to

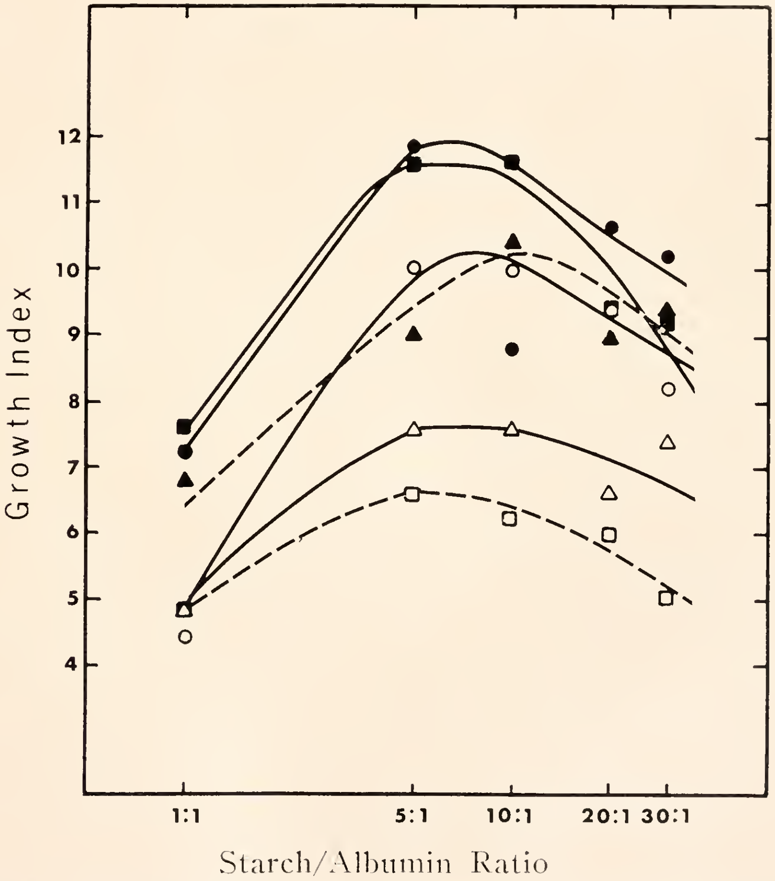

FIGURE 4. Relationships between 6 levels of total particles and varying starch per albumin ratios. Constant quantity of total particles: □ = 20 mg%; △ = 40 mg%; ○ = 80 mg%; ▲ = 160 mg%; ■ = 200 mg%; ● = 320 mg%. Conditions: Exper. 183; medium 100 (no amino acids); 5 animals in 6 ml medium; to final growth (*i.e.*, no time limit). Each point represents the average stage reached before dying by 5 animals when the total particles at its disposal were 1.2 mg (20 mg%); 2.4, 4.8, 9.6, 12, 19.2 mg.

which was added, 1 ml of the same medium, containing the desired ratio and amount of particles.

The growth index represented therefore the average growth per individual for only 6 ml medium (Fig. 4). As before the 1:1 ratio was inhibitory; the 5:1 and 10:1 ratios are optimal, and the 20:1 and 30:1 ratios are less effective. Obviously, the growth index at optimal ratios increases with the total particles offered, though the pairs 80 and 160 mg% and 200 and 320 mg% induce remarkably similar growth indexes.

As mentioned, these experiments had the objective of replacing dropwise feeding and to find ratios and total concentrations of the initial medium which would avoid mortality and permit high growth rates. Since these contrasting effects were favored by high quantities of particles, a compromise had to be made: 0.1% starch + 0.01–0.03% albumin proved satisfactory.

Replacing particles with solutes The particles employed were mixed polymers, obscuring the requirements for carbon sources and amino acids. Numerous trials to determine these requirements failed but contributed more understanding of phagotrophy and osmotrophy.

Repeated attempts to use soluble carbohydrates were done with media 39, 91 and 100. Starch was omitted, particulate proteins or albumin were used at 5–20 mg%. The carbohydrate solutions were filter-sterilized and/or autoclaved and added aseptically to the experimental test tubes. Soluble starch, dextrin, cellobiose, sucrose, galactose, trehalose, maltose, mannose, and glucose were tested in the range 0.4–4%.

Most failed to replace insoluble starch: one juvenile was obtained once in 1g% cellobiose, and a young female in 2% each of glucose + sucrose but in 3 months; a comparable female is obtained in 24–27 days on 0.1% insoluble starch. At best a 40× concentration of soluble sugars supported the same growth but in 3× the time needed for particulate starch.

From the early trials on replacing precipitated albumin with soluble mixtures of amino acids, it was evident that the amino-acid mixtures became toxic when > 0.4–0.6%, and that the nontoxic concentrations did not allow growth in the absence of precipitated protein or albumin. Since toxicity might be due to unbalances, many amino-acid mixtures were tried, including the mixtures employed for tissue cultures, for growing rats, chickens, insects, worms, and ciliates; also tried were mixtures simulating the amino-acid ratios of various proteins such as blood serum, liver, casein, albumin, globulins, and the protein composition of bacteria, yeast and algae.

These mixtures contained the 10 essential and the usual 8 nonessential. All of them became inhibitory or toxic at total concentrations of amino acids > 0.4–0.6%. [*Aedes aegypti,* a freshwater mosquito, tolerates up to 1.2% amino acid before inhibition ensues due to osmotic pressure. *Artemia* grows in 15% salts yet is sensitive to lower amino-acid concentrations.] Since at these concentrations the amino-acid mixtures could not substitute for all the albumin, further experiments were done in the presence of 2.5, 5, and 10 mg% precipitated albumin to detect partial substitution. Single amino acids and combinations of a few were also tried to find out whether albumin was deficient in any of them.

The 2 best amino-acid mixtures were AA 18L, and AA 19L (Table III): they partly replaced albumin if added to a medium 91 containing 0.1% starch + 5 mg%

Table III

Amino acids mixtures
(1 ml = mg)

Amino ac. mix / Compounds	AAP1	AAP2	AAP 18L	AAP 19L	AA 1A
L-arginine	2.6	3.0	4.6	5.7	
L-histidine	0.6		2.1	2.4	6.0
L-isoleucine	1.2		5.3	7.0	
L-leucine	1.6		9.7	9.2	
L-methionine	0.6	1.0	3.2	5.2	
L-lysine	1.4	1.0	7.6	6.3	
L-phenylalanine	1.2		5.3	7.7	3.0
L-threonine	1.0		5.1	4.0	6.0
L-tryptophane	0.3		1.6	1.2	
L-tyrosine	0.8	1.0	3.8	3.7	
L-valine	1.2		7.2	7.1	
L-cystine	0.6		2.7	2.4	
L-serine	0.6	2.0	7.9	8.2	12.0
L-proline	0.2	7.0	5.0	3.6	
L-glutamic ac.	0.6	30.0	13.5	16.5	30.0
L-alanine		15.0	5.0	6.7	
L-aspartic ac.			9.5	9.3	
glycine	0.6	20.0		3.1	
Total weight/ml	15.1	80.0	99.1	109.3	57.0
ml/100 used	5 ml	AAP$_1$ 6 ml +AAP$_2$ 1 ml	6–9 ml	3–6 ml	3.3 ml
Total amino ac.	75.5 ml	171 mg	594–891 mg	328–656 mg	188 mg
Used in medium	31	39	91	91	100

albumin: 0.3% of AA 18L replaced 3–5 mg% albumin, giving in the same time similar development. AA 19L was better: 0.33% substituted 5–8 mg% of albumin, *i.e.*, gave growth comparable to albumin 10–13 mg%. Therefore, 0.3% of soluble amino acids correspond in nutritional efficiency to 5 mg of precipitated albumin, *i.e.*, particles were ~60× more efficient than solutes.

But additions of amino-acid mixtures to a medium containing 0.1 starch + 2.5 mg% albumin stimulated growth only very slightly. This is the minimal quantity of albumin which gave some growth with or without amino acids. The complete substitution of particulates with glucose + sucrose + amino acids also resulted in no growth, with or without non-nutrient particles (see below).

However, the addition of a few amino acids (AA1A mix, Table III) to medium 100 speeded growth and gave better survival, indicating that albumin may be deficient in some amino acids.

Uptake of soluble nutrients. Failure to replace particulate carbohydrates and protein indicated strongly that *Artemia* was an obligate phagotroph. Yet paradoxically, the B-vitamins, nucleic acid components, and trace metals were offered as solutes. Still, these solutes might have become absorbed on to the particles.

This possibility was explored by preparing 2 batches of medium 91: one without vitamins, the other with vitamins (2 ml% Vit. A. VIII). The two batches were distributed in test tubes, autoclaved and centrifuged until all the particulate matter was well packed.

Both supernatants were withdrawn aseptically and passed through an ultra-fine sterile glass filter. The 2 filtrates and the 2 slurries were combined aseptically to yield all 4 possible combinations (10 ml per tube); each was inoculated with 5 nauplii.

	stage reached
1) Filtrate and particles from *no* vitamins	IV small metanauplii
2) Filtrate *no* vitamins + particles vitamins	IV medium metanauplii
3) Filtrate vitamins + particles *no* vitamins	juveniles
4) Filtrate and particles from vitamins	young adults

The results indicate that most of the vitamins remained in the filtrate and that the particles absorbed little or no vitamins. The slight difference in growth between the pairs 1, 2 and 3, 4 which might be interpreted as a very small absorption of vitamins on the particles, was probably due to the residual liquid medium which mixed with the particles in decanting.

This simple experiment resolves the apparent paradox of an animal which utilizes micronutrients as solutes yet needs bulk nutrients as particles. Inbibition occurs in *Artemia* but is so limited that only the nutrients needed as traces, and nontoxic when supplied in enormous excess, are effective in soluble form.

Effects of non-nutrient particles Several particles were tried in the hope of increasing liquid ingestion. These were tried on "juveniles" in seawater and on "young" in artificial media without starch and precipitated protein but rich in amino acids and sugars. All particles before use were ground in a colloid mill to an ingestible range (1–20 μ); all were ingested as attested by numerous fecal pellets.

Survival of "juveniles" and "young" varied depending on the particle: early mortality (*e.g.,* 8 days in seawater, 10 days in artificial media) ensued in glass, vegetable charcoal, Celite, montmorillite, graphite, Alfacell and Celloflour. Maximal survivial ((11 days in seawater, 20 days in artificial media) was induced by kaoline, Carbose, and rice starch. Cellulose, activated charcoal, and alumina gave average survival. Although 2–3 molts occurred in artificial media with the more favorable particles, the "young" did not become "young adults" except in tubes containing rice starch. The favorable action of these particles in artificial media was merely longer survival. Adsorbant particles were also tried, among them Norit A, Sephadex, and a fine powder of mixed weakly cationic and anionic resins; they were inhibitory.

Reilly (1964) reported success in eliminating the apparent requirement of *Paramecium caudatum* for the "protein factor" (a non-dialyzable fraction from dried green peas) by adding Celkate (anhydrous Mg silicate, Johns-Manville, New York) to a conventional synthetic *P. caudatum* medium to which had been added larger quantities of serine. The "protein factor" apparently both stimulated vacuole formation (the main mechanism for ingesting and digesting particles in most ciliates) and supplied amino acids suboptimal in previous media. Norit A and various starch particles were active but less effective than Celkate for *P. cau-*

datum. Adsorptive particles were also beneficial for *Glaucoma chattoni* (Holz, Wagner, Erwin and Kessler, 1961).

Celkate and Micro-cel (a hydrous Ca silicate) were tried in the artificial media containing a total of 0.72% of amino acids + 0.05% starch and 5 mg% of precipitated albumin. Celkate, although inhibitory above 10 mg%, somewhat favored growth and differentiation, giving in general one stage above the controls.

Discussion and Conclusions

The central problem in designing media was how to supply nutrients effectively. Medium 100 is almost defined; it embodies what was found about *Artemia's* needs for B vitamins, sterols, and nucleic acid derivatives. Details of these requirements will be reported separately.

Requirements for individual amino acids and carbohydrates could not be determined because the precipitated albumin and starch could not be replaced with their water-soluble ingredients. The vitamins and nucleic acids in contrast were effective as solutes.

Evidently the need for building blocks (amino acids) and energy sources—the bulk of the requirements—could not be satisfied as solutes.

These results and the experiments on the protein : starch ratios and on non-nutrient particles agree with most of the observations on the physiological behavior of *Artemia,* and indicate that it is an obligate phagotroph.

Artemia discriminates only for size and does not select from various mixed suspension of *Phaeodactylum, Chlorella, Dunaliella,* and *Arteromonas;* nor does it discriminate between plant cells and sand (Reeve, 1963b).

Indiscriminate uptake of particles may handicap phagotrophs. Growth of *Daphnia* may be impaired by non-nutritive particles in lakes turbid with fine particles of clay and silt. In trial of various mixtures of inert particles and algae, Robinson (1957) found that survival and reproduction increased at very low concentrations of inert particles, but declined as the abundance of mineral particles increased.

Equally important are variations in nutrient value of ingested algae even among species of the same genus, (from inadequate for growth, to partly or completely adequate) ; the same algal species may be adequate for one crustacean species, inadequate for another (Provasoli, Shiraishi and Lance, 1959). It is, however, this lack of discrimination that permitted replacement of live food with artificial particles of acceptable size.

Reeve (1963a) found also that *Artemia* regulates food intake by increasing ingestion rates as cell concentrations increase until a certain concentration of cells is reached; at this concentration, or above, a constant, maximum ingestion rate is maintained [*Daphnia magna* behaves similarly (McMahon and Rigler, 1965)]. In our experiments the concentration of particles was far above those used by Reeve, even at the reduced concentrations of particulates of medium 100, therefore *Artemia* was ingesting at a constant maximum rate.

Maximum ingestion rate is inversely proportional to algal cell size, thus the total volume of cells ingested at this rate remained the same for a given size of animal: 0.05 mm^3/hr/10 mm long adult (Reeve, 1963a). Hence the size variability of the artificial particles, in media 39, 91, and 100 was not important, since it was within limits of acceptability.

The difficulty in compounding artificial particulate media derives from the evolutionary adaptions of *Artemia*. Maintenance of a constant rate of ingestion in conditions of overabundance of particles, reflects the need to retain the food for the time necessary for digestion; lacking this regulation over-feeding could result in starvation due to inadequate retention time. But the retention time of *Artemia* is probably attuned to the ingestion of living cells which, upon digestion of the cell walls, become a fine suspension of organelles, colloids, and solutes—all rapidly digestible because of the enormous surface of the particles. Ingestion of live cells may be an added advantage to an organism living like *Artemia* in a hypersaline environment: the cells provide, besides nutrients, 85–90% water. *Artemia* is excellently fit to its environment: most of the few algal species living in brines are a complete food for *Artemia* (Gibor, 1956). That the retention time in the gut during maximum feeding rates is very close to the minimum time needed for digesting cells with a thin cell wall is shown by the lack of nutrient value of algae (as *Stichococcus*) which, having a thicker and probably a less digestible cell wall, pass through the gut of *Artemia* undigested and alive (Gibor, 1956).

Since Reeve (1962 *in* 1963b, p. 141) asserts that "food remains in the gut progressively longer, as there is less pressure on it from the incoming food to move through the gut," it would have been better to use in artificial media more dilute suspensions of particles: a longer stay in the gut would permit better digestion. Dropwise feeding gave faster growth, perhaps because of the far lower concentration in particles but, as mentioned, it was too laborious for aseptic work. Similarly, less particles were not employed in medium 100 because more tubes of media would have been needed to bring a few individuals to adulthood.

That time of retention, nature of particle, and limited size of the gut govern *Artemia* nutrition is indicated by the experiments with various ratios of precipitated starch and protein. Overabundance of either meant less growth or no growth (where proteins predominated), even though both particles were needed. If nutrient particles can compete for the available space of the gut, it is not surprising that non-nutritive absorbent particles had no effect on growth even though high concentrations of sugar and/or amino acids were present. Evidently very small (ground with a colloidal mill) absorbent particles, even if fully charged with nutrients, could not approach the effect on growth of like-size nutrient particles whose core and surface both were nutritive. Particles $< 1\ \mu$, or colloids undoubtedly would favor digestive efficiency (as happens when the cell wall of an alga is broken) but this was impractical because a minimal particle size is needed for full efficiency of the paropodia in sweeping the medium clear of particles and collecting them on the food groove as ingestible pellets.

Is, then, *Artemia* an obligate phagotroph devoid of osmotrophy? Since the 1959 medium provided all nutrients except starch, in soluble form we said (Provasoli and Shiraishi, 1959, p. 354) it utilized solutes for growth. We had not considered that serum is a colloid and that it could have been absorbed on to the starch particles. This conclusion was also influenced by Croghan (1958a–bcd). By ingenious, painstaking methods he found that *Artemia* regulates the osmotic pressure and ionic balance of its hemolymph by taking up NaCl and water from the gut lumen and excreting NaCl through the branchiae (metepipodites) of the first 10 pair of phyllopods (*i.e.,* swimming appendages)—the only permeable region of the whole exoskeleton.

Croghan (1958d) also kept adult *Artemia* 24 hr in seawater with dissolved neutral red. The dye rapidly colored the gut lumen, did not diffuse in the hemolymph, and became 7× more concentrated in the gut than in the surrounding medium. He concluded (p. 244) that ". . . *Artemia* rapidly swallows the medium and takes up water from it, thus concentrating the dye." The experiment of Croghan was repeatable, and allowed, in the presence or absence of particulate starch, a determination of the approximate pH of the various sections of the gut: 5.6–6.0 in the cephalic region; 6.0, 6.6, 7.0 in the thoracic region; the abdominal region remained colorless.

Our experiment showing that the B vitamins in the medium are not absorbed onto the particles and are used as solutes substantiates the conclusion that *Artemia* swallows the medium. But swallowing of medium should be minor: *a*) mixtures of sugars and complete amino acid mixtures were very poorly utilized; *b*) rapid or substantial swallowing of the highly hypersaline waters (up to 6× seawater) in which *Artemia* normally lives would impose severe stress for maintaining the hemolymph below or at the highest osmotic pressure allowable (corresponding to ~2.2% NaCl).

We must conclude that osmotrophy in *Artemia* is quite limited, and by itself incapable of sustaining growth even at concentrations of nutrients approaching inhibitory levels. In nature *Artemia* lives as an obligate phagotroph but in the laboratory it can profit in extreme conditions (very high concentrations of vitamins and nucleic acids) from its modest osmotrophy. This view is supported by the finding of Stephens and Schinske (1961) that marine Crustacea differ from all soft-bodied invertebrates in having almost no uptake of radioactive glycine.

So far, the only filter-feeders reared on artificial media are 3 mosquitoes; most work was done on *Aedes aegypti.* Singh and Brown (1957) grew it on a synthetic diet in which amino acids, RNA, vitamins, and glucose were added as solutes; the solid portion was constituted of 0.63% of finely powdered cellulose coated with a mixture of lecithin, cephalin, and cholesterol. The mixture of 17 L-amino acids was added at 1.16%. Higher concentrations were inhibitory because they exceeded *A. aegypti* osmotic tolerance (Δ 0.4 C). [The inhibitory level of the amino-acid mixtures for *Artemia* is 0.7% while the osmotic tolerance is 30% total salts!] If the amino-acid mixture was added at 0.8%, only 15% of the mosquito larvae reached the 3rd instar and none the 4th instar, indicating that even in *A. aegypti* solute efficiency is low. Also, the larval period lasted 16 days when the larvae were grown on 1.16% of the amino acid mixture (mimicking casein ratios) as compared with 7.5 days on the corresponding diet with casein.

Akov (1962), in studying antimetabolites, resumed work on *A. aegypti.* Under her conditions she found that the amino-acid mixture of Singh and Brown was extremely inefficient [median time to pupation (MTP) was 45 days; only 15% became adults.] If 0.05% of finely powdered (80 mesh) dry-sterilized casein was added to the amino-acid mixture, 95% of the larvae reached adults; the MTP was 10 days. The inefficiency of the solutes was clearly shown when casein hydrolysate was compared with solid casein: with 2% of the hydrolysate 12 days were needed for pupation and 60% of the larvae became adults; with 0.1% casein, median time to pupation was 6.5 days and 90% adults were obtained!

Thus even for *A aegypti,* a filter-feeder which can be grown on solutes, phagotrophy is by far the most efficient source of building blocks and energy.

An important difference between *Artemia* and insects is that casein (or amino-acid mixtures mimicking it) seem well balanced for all insects so far investigated, while it is ineffective or toxic for *Artemia* when powdered and dry-heat sterilized; several samples were tried with similar results. Many other proteins besides egg albumin were dissolved and heat precipitated at their isoelectric point, but unfortunately these particulates redissolved when autoclaved in the alkaline medium needed by *Artemia* (*Artemia* cannot grow below below pH 6.8 in seawater or our artificial seawater media).

Another difference is that *A. aegypti* did not need any carbohydrate when the solid casein was supplied at 1%. *Artemia,* on the contrary, needed ratios of 10:1–2.5:1 for starch-albumin; albumin alone was toxic. This need for a preponderance of starch could be an artifact since the composition of 10 marine algae tested by Parsons, Stephens and Strickland, (1961) is similar: carbohydrates 15–30%, proteins 36–57%, fats 3–10%. It might however be an Artemia peculiarity since *Daphnia magna* raised on similar artificial media likes a predominance of protein.

Preliminary work on *D. magna* indicates also that the particulates are as highly efficient as in *Artemia. Daphnia* was brought in aseptic culture as a comparative organism for deciding whether phagotrophy in *Artemia* was not due to its need to avoid swallowing hypersaline media. *Daphnia,* living in fresh waters, has no need to avoid swallowing, and being able to grow in highly organic solutions of dung (Banta medium) should be more resistant to organics.

Phagotrophy being the most efficient system, the problem of designing artificial media for filter-feeders centers in supplying all the nutrients, so far as possible, as particulates.

SUMMARY

1. The 1959 undefined artificial medium for *Artemia* was simplified to a medium containing only defined ingredients: a liquid phase containing mineral salts, 6 amino acids, 5 nucleic acid components, 8 vitamins, 2 sugars, a pH buffer, and a fine particulate phase consisting of precipitated albumin, gelled rice starch, and cholesterol. The amino acids and sugars are dispensible.

2. Starch and albumin were not replaceable by their soluble components (sugar and amino acids) even in the presence of inert or absorbing particles. Phagotrophy appeared the most efficient way to satisfy the bulk nutritional requirements.

3. Growth rate and differentiation depended upon starch:protein ratio and total quantity of particles.

4. *Artemia* ingested liquids but apparently to a very limited extent since vitamins and nucleic acid components (nontoxic even at very high concentrations) were utilized as solutes only when in high concentrations. Amino acid mixtures on the other hand became toxic at concentrations too low to satisfy growth requirements.

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THE EFFECT OF PREGNANCY ON PERIPHERAL BLOOD IN THE MOUSE

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The increasing demands by the growing mammalian fetus upon the maternal tissues for food and oxygen would be expected to cause significant changes in the maternal peripheral blood cell counts during pregnancy. So-called "physiological anemia" has been reported in the rat, rabbit, and in man (Zarrow, 1961) but this may be misleading because of the lack of correlation between plasma volume and red cell volumes (Fruhman, 1968). The low hemoglobin levels per unit volume of peripheral blood in human pregnancy is due to a greater increase in total blood volume (30%), plasma volume (40%) than to an increase in red cell mass (18%) (Low, Johnston, and McBride, 1965). Nevertheless peripheral blood changes as reflected in total blood counts associated with the progress of a pregnancy certainly reflect the increasing fetal demands upon the maternal circulation, so that the following study was designed.

Materials and Methods

CF1-S mice used for this study had been matured by being put through a single pregnancy. Thirty mice were time-mated to 45 minutes so that their gestational ages were identical. On each alternate day from day of conception through day 18, fifteen mice were blood checked. Delivery normally occurs on the 19th day after conception. The small amount of blood removed from the tail tip on alternate days was believed to be completely replaced within the 48 hours before the next bleeding. Thus the data given are averages of 15 normal but pregnant mice for each gestation day.

Blood was always taken before noon of the day designated in order to avoid any diurnal shift in the normal counts (Urushijama, 1959). Fetal blood taken on gestation day 17 was removed quickly with pipettes and rubber tubing all ready. The head of the fetus was held in the left hand, the lower part of the abdomen being immobilized by the thumb of the same hand. The jugular vein was plainly visible through the thin fetal skin, and this was pierced with scissors. All counts were based on a minimum of 15 mice of each sex (Rugh and Somogyi, 1968ab).

A complete analysis of the blood smears was made for the 17 gestation day mouse fetus, to compare its varied cells with those found in the maternal peripheral blood. Examination could not be made before the 17th day simply because it was not possible to obtain from earlier fetuses enough whole blood. It was, however, possible to sex mice at 17 days gestation so that the data are divided as to the two sexes and comprise averages of 15 such fetuses. To make a further comparison, blood data were collected from 25 separate 9 week old mature adults of both sexes.

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[1] Based upon work performed under Contract AT-(30-1)-2740 for the U. S. Atomic Energy Commission.

It is known that there are daily rhythms in the various peripheral blood cell types in normal animals so that this variant was avoided by taking all blood samples at the same time of day, namely in the morning (Pauly and Scheving, 1965). There are also variations in blood counts among closely related strains of mice (Russell, Neufeld and Higgins, 1951; Pauly and Scheving, 1965), and even sex differences occur (Halberg, Hammerston and Bittner, 1957). This study was based entirely upon the CF1-S strain, and the sexes were distinguished for all tests including those of the 17 day fetus (Rugh 1968).

Experimental Data

The daily weights were taken of the impregnated mice. The three month old female mice averaged a little less than 25 grams at the time of mating. This average dropped a few grams at the second day. There was then a slow climb in average total body weight when at about 13 days of gestation all mice gave external evidence of their pregnancies and the weight average had increased from about 25 to about 34–35 grams. There followed a steady daily increment in average body weight to a maximum at 18 days (Fig. 1). The average gain in weight before birth was about 22 grams, almost a doubling of the original body weight. This dropped after delivery of the litter to an average total weight of about 31 grams or still some 6–7 grams above the pre-conception weight. This residual weight gain was probably due to mammary activity. Female mice after an initial pregnancy, no matter at what age it occurs, tend to remain heavier than they were prior to that pregnancy.

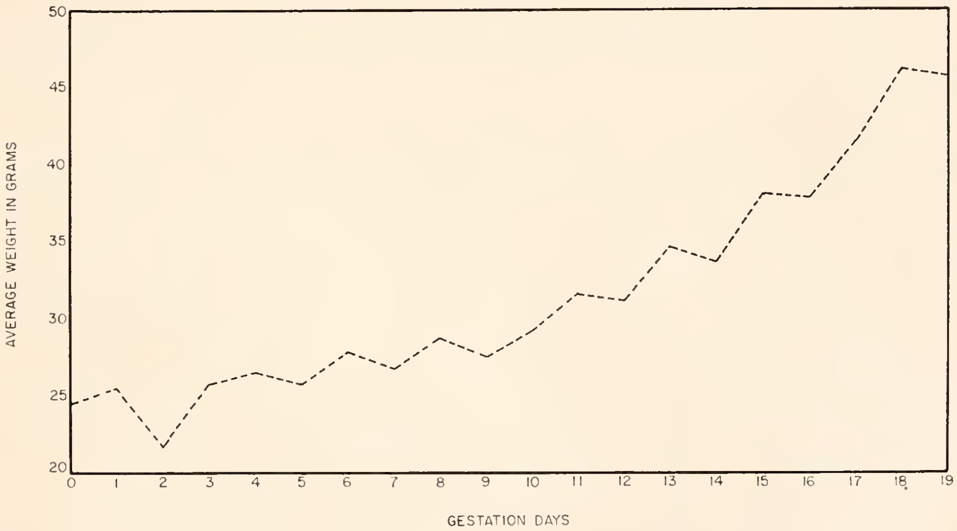

Figure 1. Average weight changes during pregnancy in mice.

The average peripheral red blood cell counts of the 25 control 9 week old female mice was 9,700,300 and the initial counts of the impregnated mice was close to this. The accompanying graph shows that there was an immediate but slight drop in the red blood cell count by 1 day after conception, with a further drop developing after day 5 and continuing until just before birth. Maternal blood at parturition was relatively anemic but not seriously so. This anemia might be caused by an avidity on the part of the fetus, since pregnancy in humans is often associated with anemia. The steady drop in red cells began at about 5 days. This is the time when the maternal-fetal relations become quite intimate, following implantation on day 4½, so that decidual hemorrhage may have played some part in the mild maternal anemia (Fig. 2). Maternal blood never becomes seriously anemic since the lowest point, at 17 days, was still about 6,500,000 red cells.

The average white cell counts in the maternal peripheral blood showed two prominent peaks, at 3 and at 9 days. These are probably best explained on the basis of changes in the fetal-maternal relationships. At 3 days the developing morula-blastula stage move through the oviducts into the uteri and as foreign and invading entities they are probably irritants causing some local leukocytosis. This has been observed in sections of the uterus just prior to implantation (Rugh, 1968). It is also possible that the response of the leukocytes to the invasion of the female reproductive tract by sperm cells is relatively slow and the peak of this response seems to persist for about three days after the invasion by the sperm. Such phagocytosis of sperm is regularly seen in post-conception uteri in the mouse. The average white blood cell count of the impregnated mice on conception day was considerably lower than the average for similar, but non-pregnant 9 week old mice and was slightly above 7000. There was a transient but nevertheless real elevation of white cells to about 9800 by 3 days, being the average of 15 mice simultaneously

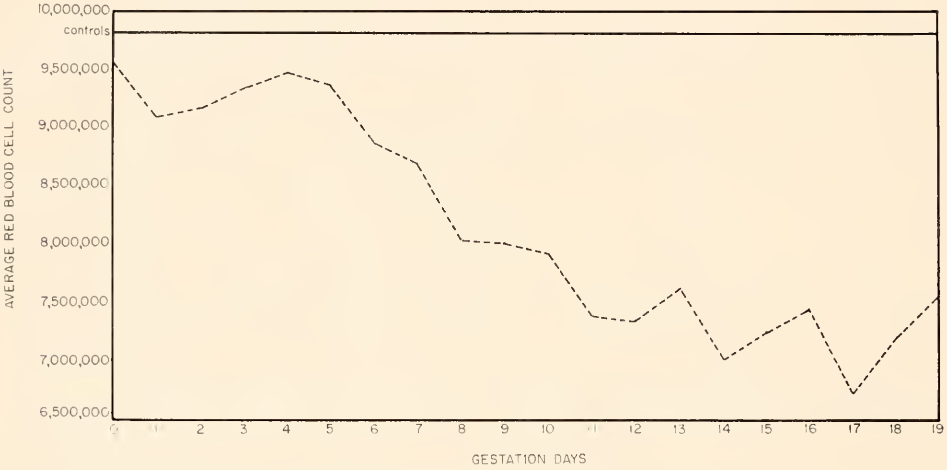

FIGURE 2. Average red blood cell count changes during pregnancy in mice.

impregnated. The white cell count then dropped slowly to the 7th day almost to the pre-conception level, but then rose rapidly in two days to another high at 9 days. This second rise could be explained on the basis of placenta formation which begins between 7–8 days and is very active on days 9 and 10. Such an invasion of maternal tissues of the uterus by the fetus, and penetration by fetal villi, could easily cause a rise in the counts of the protective white blood cell elements (Fig. 3). As soon as the placenta was established the white count dropped to its lowest point at 13 days, to remain sub-normal for the duration of the pregnancy. Thus there appears to be a complete adjustment of the maternal blood elements to the invasion by the fetus of the maternal tissues and the white cell count remained considerably below the infection level.

The tabular data from the 17 day old fetus, based upon averages of 15 males and 15 females, is of interest particularly in relation to the peripheral red and the white cell counts. These are shown in Table I where the data are also given for the same strain of mice (average of 25 mice for each figure) at 9 weeks of age. It will be noted that the hemoglobin percentage is considerably lower than for the adult controls, and there persist some immature forms. The red cell counts of the 17 day fetuses were about 35% and the white cell counts about 11% that of the adult controls. Platelet counts were also about 33% those of the control adults. Thus, hematopoiesis was not yet adequate to bring the 17 day mouse fetus up to the adult level so that the late mouse fetus must still be dependent upon the mother for oxygen and protection. Among the white cells the lymphocytes appeared to be making greatest proliferative increase at this time, being about 50% of the count found in the adult controls. The neutrophils (except for the stabs) occurred in about the same proportion as in the adults.

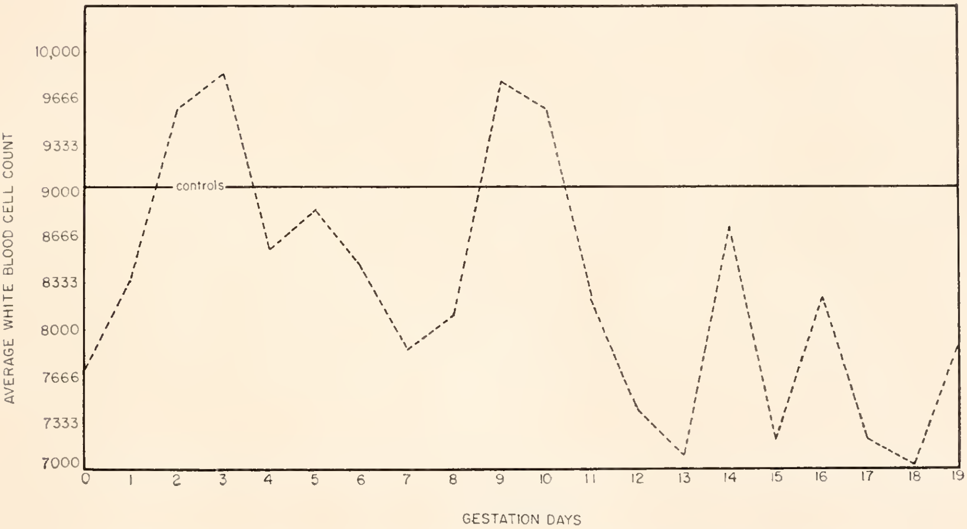

FIGURE 3. Average white blood cell count changes during pregnancy in mice.

TABLE I

Blood analysis of fetal and adult mice

	Fetuses at 17 days		Adults at 9 wks	
	Males (15)	Females (15)	Males (25)	Females (25)
Hemoglobin	8.9	8.3	14.1	15.1
RBC (Erythrocytes)	3,438,666	3,244,000	9,140,800	9,700,300
WBC (Leukocytes)	1,720	1,533	10,520	9,976
Platelets:	453,333	454,000	1,361,600	1,342,000
Neutrophils:Segmented	21.9	25.1	27.1	23.1
Neutrophils:Stabophils	10.3	7.1	1.1	0.4
Neutrophils:Metamyelocytes	1.5	0.8	0.0	0.0
Neutrophils:Myelocytes	0.1	0.1	0.0	0.0
Eosinophils:	2.5	1.0	3.5	4.1
Eosinophils:Meta	0.5	0.0	0.0	0.0
Monocytes:	18.7	19.7	5.8	4.3
Monocytes:Young	0.9	1.1	0.0	0.0
Lymphocytes:	31.8	30.4	61.5	67.0
Lymphocytes:Young	2.1	2.4	0.2	0.1
Blasts:	0.5	0.8	0.0	0.0
Histiocytes:	0.3	0.1	0.0	0.0
Phagocytes:	0.4	0.7	0.0	0.0
Granular-Vacuolated Cells:	0.9	4.1	0.0	0.0
Early Type Ring Nuclear Cell:	5.3	4.6	0.04	0.1
Double Nucleated Lymphocytes:	0.1	0.1	0.5	0.5
Nucleated Erythrocytes:	48.6	53.7	0.0	0.0
Unidentifiable Cells:	2.0	0.4	0.0	0.0

Young Lymphocytes: stage between blast and adult (one without nucleoli and less blue cytoplasm)

Histiocytes: a very early cell with no phagocytized particle but less granules.

DISCUSSION

Accelerated erythropoiesis does occur in the pregnant mouse (Fowler and Nash, 1967), but in the fetus the first blood cells to appear do so at about 7 days (Rugh, 1968). There is evidence of the erythropoietic initiation independent of the mother (Jacobson, Marks and Gaston, 1959). The yolk sac hematopoiesis provides the primitive generation of erythrocytes which are exceptionally large, nucleated cells. As early as 13 days gestation the nucleated erythrocytes begin to disappear, but as late as 17 days some of these miniature red cells may be seen. The liver, spleen, and bone marrow begin their active hematopoiesis when the circulatory system is closed and functioning by 9 days gestation. Thus, the fetus, independently of the mother, rapidly forms its own circulatory and hematopoietic system, with myelopoiesis starting about day 15.

While mouse development is telescoped into about 19 days, there is relatively little involvement of the maternal tissues until implantation (about day 4½). The sudden and drastic rise in the white count at 3 days can be explained only on the basis of a defense reaction by the maternal tissues against the invasion and irritation of the genital tract by the relatively high volume of semen and spermatozoa. Histological sections of the uterus at this time show massive migration of leukocytes toward the lumen, and actual phagocytosis of the spermatozoa so that relatively few of them survive to reach the ampulla for fertilization.

A recent study (Fruhman, 1968) indicated that the maternal spleen is the primary organ of erythropoiesis in the mouse, and it was this organ that showed the most striking changes during pregnancy. The reason for such changes in the spleen was believed to be the increasing oxygen requirements of the fetal tissues. Since litter numbers vary from 1 to about 20, it is simple to imagine that the degree of tissue hypoxia may be varied in direct relation to the numbers of viable fetuses. As the fetuses increase in total volume this increased hypoxia could readily affect both fetal and the maternal hematopoietic organs. After the 5th day of gestation the red cell counts dropped steadily reaching a low just before delivery, so that both mother and fetus were anemic. This suggests that the maternal hematopoietic organs did not respond to hypoxia by accelerated hematopoiesis but that the mother's red cell counts changed toward the level of anemia while the fetus was accelerating hematopoiesis. The mother showed a steep incline toward recovery in red blood cells as early as 18 days, when the fetus normally begins to become functionally independent. Blood volume studies were not made. Hypoxia, therefore, did not stimulate maternal hematopoiesis but may be related to fetal hematopoiesis.

SUMMARY

1. Fifteen pregnant mice, time-mated so as to be simultaneously at the same stages of gestation, were weighed daily at the same time for the duration of their pregnancies. As expected, except for gestation day 2, there was a steady increment to 45 grams in the average total body weight of the 15 pregnant mice at day 18, as the fetuses are preparing for birth. The average total body weight almost doubled the original body weight of 25 grams.

2. The white blood cell counts of the pregnant mouse showed a rapid rise by the third day, possibly related to marshalling of leukocytes (neutrophils and macrophages) to phagocytize the invading spermatozoa, or to changes in the uterine mucosa anticipating implantation which usually occurs at day 4½. There is a second peak in the peripheral white cell count at 9 days when the placenta is being organized. Thereafter the average white cell count remains below the normal level for this strain of mouse, with a slight rise of the average at 14 days and another at 16 days, but neither peak bringing the count up to normal.

3. The maternal erythrocyte count showed a steady decline almost from the day of conception, approaching the level of anemia but indicating possibly that the growing fetuses were depriving the mother of nutritive elements necessary for erythropoiesis.

4. The fetal blood at 17 days gestation, two days before birth, showed average deficiencies in the hemoglobin, red and white cell counts and in platelets. Adult type lymphocytes were about 50% of the adults and nucleated reds were still abundant.

5. In general there was evidence of a decreasing average of peripheral erythrocytes in the blood of pregnant mice as gestation progressed while the white cells showed several peaks of production probably related to major structural and functional changes of the intimate relation between fetus and mother. These struc-

tural changes related to implantation, placentation, and the functional changes to increasing hypoxia in the crowded condition of the gravid uterus.

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LIGHT TRANSMISSION AND SPECTRAL DISTRIBUTION THROUGH EPI- AND ENDOZOIC ALGAL LAYERS IN THE BRAIN CORAL, *FAVIA*[1]

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Two species of algae were living symbiotically with a hard brain coral, *Favia,* harvested in the environs of the Flinders Island Group on the Great Barrier Reef in Australia. One of the algae has the microscopic appearance and pigment composition of dinoflagellates (Halldal, 1968, Jeffrey and Haxo, 1968) and it contributes the dark brown color to the coral tissues which form a surface layer over the colony. This alga resembles *Symbiodinium microadriaticum* Freudenthal. Green algae lived inside the spherical coral forming another colored layer (Jeffrey, 1968), and there was an intermediate pale green (nearly white) layer between these brown and green layers. The green algae seemed to be of mixed genera and species, most of them probably belonging to *Ostreobium Reineckei* Bornet within the order Siphonales. The present paper describes the *in vivo* absorption spectra of these algal layers as well as the spectral distribution.

EXPERIMENTAL METHODS

The samples of *Favia pallida* Dana were harvested in the environs of the Flinders Island Group, and were kept in running sea-water aquaria on the open deck of the research vessel. The spectral data presented in this paper were observed for the sample which was 12 cm in diameter and 8.5 cm in thickness (Fig. 1 left). The coral colony had a brown layer of 4 mm thick, a green layer of 2 mm and an intermediate layer of 9 mm between them. A square piece of about 4.2 × 4.2 cm^2 in surface area was cut out of this sample with an electric sawing machine, and the white lime below the green layer was ground off to obtain a piece with the three layers such as shown on the right side of Figure 1. The piece with the three layers was further cut or ground to obtain a sample of a single or double layer according to experimental requirements.

Spectroscopic measurements were carried out on the research vessel with a Shimadzu Multipurpose recording spectrophotometer model MPS-50. This spectrophotometer designed by Shibata, one of the authors, had two photomultipliers with the end-on type of photocathode, one for the sample and the other for the reference, and was suitable for the measurements of translucent and dark pieces of corals for the following reasons. a) The double detector system makes it possible to read a high absorbance value accurately because of the absence of interaction (cross talk) between the separate electric channels from the two photomultipliers. In ordinary recording spectrophotometers with a single photomultiplier, the cross talk between alternative sample and reference signals (photocurrents) from the single detector introduces systematic errors to the absorbance reading when the reading is high. b) With the spectrophotometer, absorbance can be read up to 3 by electric amplification, and the use of a light attenuator for the reference beam together with this electric amplification enables us to measure an absorbance value as high as 6. c) The end-on photocathode captures all of the light transmitted through a piece of coral placed close to the photocathode, so that the reading is an absolute value of semi-integral attenuance (Shibata, 1958 and Shibata, 1959) necessary to calculate the transmittance through such translucent samples (Shibata, Benson and Calvin, 1954). This contrasts with the measurements with the side-on type of photomultipliers commonly used. The small photocathode inside the tube of the side-on type captures only a small fraction of the diffuse transmitted light, and the fraction varies, depending on the optical geometry of the instrument and the angular distribution of the diffuse transmitted light (Shibata, 1959). When such a detector is used in making measurements on translucent samples, quasi-attenuance (Shibata, 1959) is measured rather than absorbance or semi-integral attenuance (Shibata, 1959). The light transmitted through a heterogeneous translucent sample is generally composed of different kinds of light with different angular distributions of intensity. A typical example is the light transmitted through a cell suspension which is composed of completely parallel light transmitted through the suspending medium and diffuse light transmitted through the cells in suspension. The measurement at a distance from a heterogeneous sample captures different fractions of these different light fluxes, so that the spectrum thus observed in terms of quasi-attenuance is greatly distorted as demonstrated previously (Shibata, Benson and Calvin, 1954, Shibata, 1957, and Shibata, 1958). d) The photocathode of the end-on photomultiplier, Shimadzu R-236, developed for this spectrophotometer is more red-sensitive than ordinary photocathodes. A high resolution was thereby obtained in the spectral region of

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[1] The present study was carried out in cooperation with Drs. P. Halldal and S. W. Jeffrey on the research vessel, R. V. ALPHA HELIX, of University of California during the expedition in 1966 to the Great Barrier Reef, North Queensland, Australia, and was supported by the National Science Foundation of the U. S. A.

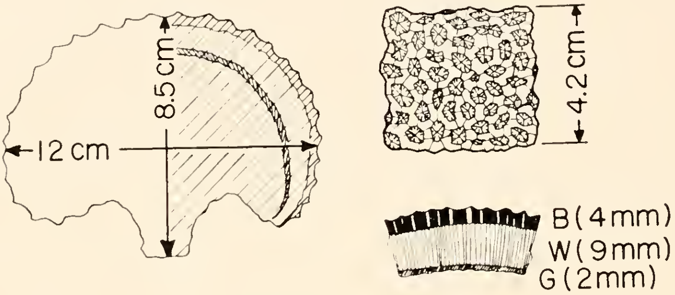

FIGURE 1. The sample of *Favia*; the dimensions of a vertical radial section (the left figure) and a piece cut for spectroscopic measurement (the right figures) as seen from above (upper figure) and again in vertical section (lower figure). B, W and G stand for the brown, the intermediate white and the green layers, respectively.

the red bands of chlorophylls without sacrificing much sensitivity in other spectral regions.

Fluorescence action (or excitation) spectra were observed with the same spectrophotometer, using the fluorescence attachment model I. A red filter, which cut the light below 660 mμ, was used for the measurement of the red fluorescent light of chlorophyll *a*.

Results and Discussion

The absorption spectrum of the brown layer of 3 mm thick is shown by curve A in Figure 2, where the absorbance value at 800 mμ is taken to be zero; the spectrum in absolute units is shown in Figure 3. The spectrum shows the red band of chlorophyll *a* at 678 mμ and its Soret band around 430 mμ, both being in agreement with the maximum wavelengths obtained previously for intact green leaves and algae (Shibata, Benson and Calvin, 1954, Shibata, 1957 and Shibata, 1958). In addition to these maxima, the spectrum shows maxima and shoulders at 635, 620, 590, 540, 500, 470, and 430 mμ. The two bands at 635 and 590 mμ are interpreted as the bands of chlorophyll *c* (Jeffrey, 1963, Jeffrey and Allen, 1964, Jeffry and Haxo, 1968, Jeffry and Shibata, 1969, and Shibata, Benson and Calvin, 1954), and the band at 620 mμ may be the second red band of chlorophyll *a*. A

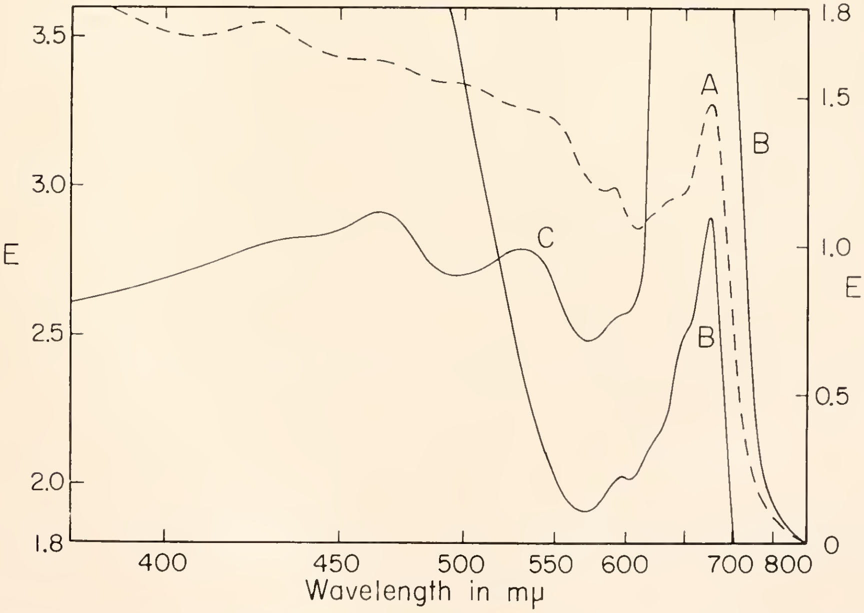

FIGURE 2. The *in vivo* absorption spectra of the brown layer of 4 mm thick (curve A) and the green layer of 2 mm thick (curve B), and the fluorescence action spectrum of the brown layer (curve C). The absorbance value, *E* (more correctly speaking, the semi-integral attenuance, $_{p}E_{t}$; Shibata, 1959) at 800 mμ for curves A and B are taken to be zero, and the action spectrum is shown in arbiturary units. The scale on the left side of the figure is for curve B above $E = 1.8$, and that on the right side is for curves A and B below $E = 1.8$.

characteristic round band at 540 mμ in this spectrum may be ascribed to peridinin (Jeffrey and Haxo, 1968), and the wavelength is in agreement with that of a peak found in the photosynthetic action spectrum of the same alga taken from the brown layer (Halldal, 1968). The bands at 430 and 470 mμ may be due to chlorophylls *a* and *c*, respectively, and a band at 500 mμ may be a composite of carotenoid bands (Jeffrey and Haxo, 1968). The photosynthetic activity versus light intensity was measured by Halldal (1968) for the same alga. The photosynthetic activity curve thus measured with the light at 440 mμ indicated a linear relationship below the intensity 1500 erg/cm^2 sec, saturation at 95,000 erg/cm^2 sec and no inhibition of activity at 225,000 erg/cm^2 sec which is approximately equal to the sun light energy available for photosynthesis below 700 mμ on a sunny day on the earth.

Curve C in the same figure shows the fluorescence action (excitation) spectrum obtained for the alga taken from the brown layer. The two distinct bands at 465 and 530 mμ indicate contribution of both peridinin and chlorophyll *c* to the fluorescence of chlorophyll *a*. This implies a transfer of energy from these pigments to chlorophyll *a*. The band at 500 mμ in the *in vivo* absorption spectrum is lacking in the action spectrum. This indicates the transfer of little or no energy from the carotenoids having an absorption maximum at this wavelength.

The spectrum of the green layer of 2 mm thick is shown by curve B which indicates the absorption characteristics of the green algae; red bands of chlorophyll *a* at 678 mμ and 620 mμ and a shoulder of chlorophyll *b* at 650 mμ. The shoulder of chlorophyll *b* at 650 mμ is more distinct than in *in vivo* spectra of common green algae such as *Chlorella* and *Scenedesmus*. The higher content of chlorophyll *b* in the green algae was reflected in the photosynthetic action spectrum observed by Halldal (1968), and agrees with the observation by Jeffrey (1968) that the content of chlorophyll *b* is about $\frac{2}{3}$ of the chlorophyll *a* content. The *a/b* ratios determined by a new precise method for several species of common plants and algae are very close to 3 (Ogawa and Shibata, 1965). The small peak at 595 mμ may be ascribed also to the high content of chlorophyll *b*. The spectrum in the Soret region shows a chlorophyll *a* band around 430 mμ and a round shoulder around 470 mμ as shown by curve B in Figure 3. Curves A and B in Figure 3 are the same spectra as curves A and B in Figure 2, respectively, but shown in absolute values (E) of semi-integral attenuance, the attenuance in terms of total transmitted light (Shibata, 1959). It is clear from these curves that the thinner green layer absorbs light more strongly than does the brown layer.

Favia has a brain-shaped surface with many ridges as shown on the right side of Figure 1. The dinoflagellates live in tissues of the coral between the ridges, and each dark brown area surrounded by ridges was 3 to 6 mm in diameter. When we looked at a lamp through a piece of the brown layer from its inside, we could recognize two kinds of light transmitted through the layer; brown dim light through the algal part between the ridges and nearly white light through the ridges. Therefore, the reading of attenuance of a single brown layer was considerably dependent on the area to be measured. On the other hand, the spectrum of a double layer composed of the brown and the intermediate layers was less sensitive to the area to be measured. This seemed to be due to the light-diffusing effect of the thick intermediate layer which will homogenize the two kinds of light transmitted through the heterogeneous brown layer. The spectrum of a piece of

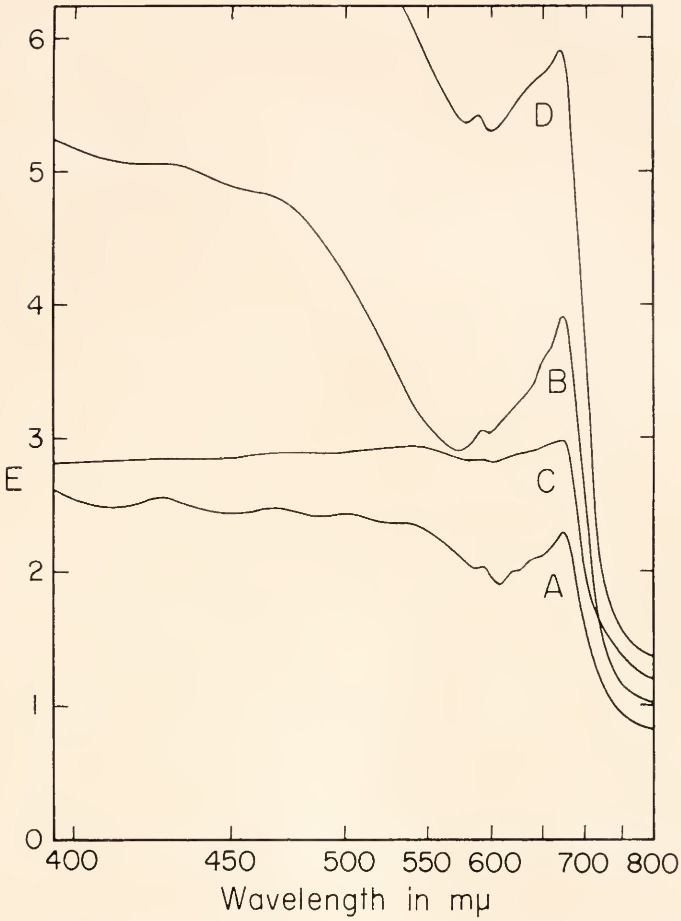

FIGURE 3. The *in vivo* absorption spectra in absolute units of semi-integral attenuance, $_pE_t$, of a brown layer (curve A) of 4 mm thick, a green layer (curve B) of 2 mm thick, a double layer (curve C) composed of the brown and the intermediate white layers (13 mm in total thickness) and a triple layer (curve D) composed of the brown and the intermediate layers and one half (1 mm) in thickness of the green layer.

the double layer is shown by curve C in Figure 3, which indicates practically no change of attenuance below 678 mμ. This implies that the white light transmitted through the ridges predominates in intensity over the brown light transmitted through the algal part. The attenuance values read between 400 and 678 mμ ranged from 2.82 to 2.99 which corresponds to 0.10–0.15% in transmittance. The sun light intensity on the earth may be about 100,000 lux on a sunny day in the tropical area which is roughly 500,000 erg/cm^2 sec in terms of the energy available for photosynthesis below 700 mμ. The above attenuance values, therefore, indicate that the light falling on the upper surface of the green layer is 100–150 lux or 500–750 erg/cm^2 sec as the energy available for photosynthesis. Halldal (1968) found that the alga taken from the top of the green layer shows linear response with intensity to 1000 erg/cm² sec at 440 mμ, saturation between 1000 and 1500 erg/cm² sec, and no photosynthetic inhibition at 5000 erg/cm² sec. The fact that the intensity on the upper surface of the green layer is slightly though not much lower than the saturation intensity suggests that the attenuation of light through the double layer is significant. The light below 700 mμ required for photosynthesis comes mostly through the ridges, and the ridges in combination with the intermediate white layer thus works as a gray filter for the green alga to bring about photosynthesis under an optimum dim light condition. The attenuance value on curve C drops steeply above 678 mμ. Above this wavelength, the light transmitted through the brown algal part between the ridges predominates over the light transmitted through the ridges, and this phenomenon seems to be responsible for the great drop of attenuance on curve C (Fig. 3). The attenuance values read at 700, 720, and 800 mμ on this curve were 2.23, 1.67 and 1.20.

Curve D in Figure 3 is the spectrum of a triple layer composed of the brown and white layers and about one half the thickness of the green layer. The attenuance value on the curve is 5 to 6 between 550 and 680 mμ and is higher than 6 below 550 mμ. This indicates almost complete absorption of the weak light by the green layer. The spectrum of a piece of a complete triple layer could not be observed directly because the attenuance was too high. The spectrum may, however, be calculated as the sum of curves B and C in Figure 3, since the effect of the multiple reflection between the green and the white layers may be assumed to be negligible when we have such a dark green layer on one side (Shibata, 1959). The attenuance values of the complete triple layer thus calculated at the Soret maximum, 430 mμ, at the minimum, 580 mμ, and at the red maximum, 678 mμ were roughly 8, 6 and 7, and those at 700, 720 and 800 mμ were 4, 5 and 2, respectively.

These data indicate that the light intensity in the middle of the green layer is roughly 0.5 to 5 erg/cm² sec between 550 and 680 mμ, and less than 0.5 erg/cm² sec below 550 mμ, and that the intensity reaching the bottom of the green layer is 0.005, 0.5, 0.05 and 5000 erg/cm² sec at 430, 580, 678 and 800 mμ, respectively. The photosynthetic activity observed by Halldal (1968) for the cells taken from the middle of the green layer showed linear response with intensity to about 100 erg/cm² sec, saturation between 100 and 700 erg/cm² sec, reduced activity between 700 and 1800 erg/cm² sec and photooxidation above this intensity. The low light intensity in the middle of the green layer allows such cells to grow without inhibition. On the other hand, the cells taken from the bottom of the green layer showed photooxidation at all intensities applied (Halldal, 1968), and the pigments extracted from the deep area contained decomposition products of chlorophylls, which suggests that the cells eventually become moribund with consequent decomposition of chlorophylls (Jeffrey, 1968). The very low light intensity reaching the bottom of the green layer is sufficient to cause the photooxidation observed *in vitro* by Halldal (1968). Another fact to be noted (Halldal, 1968) is that a shoulder appears at 720 mμ in the absorption spectrum when the green layer is kept in darkness. The formation of this band related to the high light transmission at 720 mμ, which is approximately 2%, has been discussed in his paper.

Coral colonies contract their polyps when removed from the sea, and the contraction exposes the ridges to sunlight while polyps expanded under the sea cover the ridges. Therefore, the spectra observed in the present study for the contracting state may be different from those in the expanding state. The effect of this expansion will make the absorption bands higher and sharper as expected from the theory and experiments of the flattening effect (Duysens, 1956, and Itoh, Izawa and Shibata, 1963), which is the spectral change due to localization of pigments and is generally smaller in order than the artifact caused by measuring the quasi-attenuance of heterogeneous translucent samples by a common technique. The flattening effect in this case may be appreciable, but will not change the orders of the light intensities in corals discussed above.

SUMMARY

Two species of algae are living symbiotically with a hard brain coral, *Favia pallida;* a brown alga resembling *Symbiodinium microadriaticum* Freudenthal as a brown surface layer and green algae, most of them probably belonging to *Ostrebium Reineckei* Bornet, as a green layer with an intermediate white layer between them. The *in vivo* absorption spectra of these algal layers were observed with a new spectrophotometer suitable for the measurements of translucent dark pieces of corals, and light transmission through these layers of algae and its spectral distribution were calculated from the spectra. The spectrum of the top brown layer showed the band characteristics of dinoflagellates, and the *in vivo* bands of chlorophylls *a* and *c* and peridinin were identified. The bands of chlorophyll *c* and peridinin were also found in the fluorescence action spectrum which indicates energy transfer from these pigments to chlorophyll *a*. The spectrum of the third layer showed the band characteristics of green algae, and the light intensity and spectral distribution after transmission through the brown and the intermediate layers were found to be suitable for the green alga on the top of the green layer to bring about photosynthesis actively. On the other hand, the light intensity in the middle or at the bottom of the green layer was very low, being consistent with the fact that the pigments of the cells in these deep areas are easily photooxidized.

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INTERTIDAL ZONE-FORMATION IN *POMATOLEIOS KRAUSSII* (ANNELIDA: POLYCHAETA)[1]

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Pomatoleios kraussii forms a well-defined intertidal zone in many areas of its Indo-Pacific distribution (for details of this distribution see Straughan, 1967a). However, Straughan (1968) noted that this species settles and survives subtidally, and in artificial habitats (for example water cooling systems) that are continually submerged. Hence the intertidal distribution of *Pomatoleios* is not the result of differential larval settlement. The following study was designed to determine the factors contributing to the formation of an intertidal zone by *Pomatoleios* in Hawaii.

PHYSIOGRAPHY

Coconut Island is situated in Kaneohe Bay on the northeastern (windward) coast of the island of Oahu (21°26′N, 157°48′W). It is a small, partially artificial island surrounded by a reef flat of mainly dead coral. Experimental studies were conducted on the protected side of the island—furthest from the open sea.

Temperature

Over a one year period, May 1967 to May 1968, surface water temperatures varied between 19° and 28° C, and remained above 25° C from May to October (Bathen, 1968).

Salinity

A number of streams discharge into Kaneohe Bay. During wet winter months, and particularly following heavy rain, the bay fauna is exposed to salinities somewhat lower than normal seawater. Bathen (1968) reported salinities of 35 to 36‰ for eight months of the year with a minimum salinity of 31‰ in November.

Water movement

Surface currents across the reef and inshore of the island are dependent on tide and wind. Bathen (1968) reported that the north-northeast Trade Winds increase surface currents on rising tides and decrease currents on falling tides; whereas the southern Kona Winds (November through April) may have the reverse effect. The tides are mixed but the days on which there is only one tide are limited to two or three a month.

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Northeast Trade Waves (4 to 12 feet high) enter Kanehoe Bay for 90 to 95% of the time during summer and 55 to 60% of the time during winter. At other times during winter, the North Pacific Swell (8 to 14 feet high) predominates (Moberly and Chamberlain, 1964). During the present study, the latter commenced to influence the effective sea level on September 17 when the surf level rose on the north shore of Oahu (Dr. Jeannette Whipple Struhsaker, personal communication). Although this effect is damped in the Bay, a rise in effective sea-level is still evident during October. The theoretical Mean Sea Level is 1.0 to 1.2 feet above Datum.

Material and Methods

Temperature

Recorded by continually exposed maximum-minimum thermometers (accurate to 0.2° C) placed at the same level as intertidal populations of *Pomatoleios* on both the eastern and western sides of Stations C and G. Hence they recorded both water and air temperatures to which the population was exposed.

Salinity

Measured periodically with a refractometer accurate to 0.5‰.

Water movement

Surface water currents were gauged timing floats over measured distances, under calm conditions, at midflood and midebb of the tide when the tidal ranges were 1.6 feet and 1.8 feet, respectively. Measurements were made at inshore and reef flat stations.

Distribution and abundance of Pomatoleios

A. Distribution around the coast of Oahu: In June and July the following localities were surveyed for *Pomatoleios*: Kahuka, Koko Head, Black Point, Kewalo Basin, Nanakuli, and Maille Point, as well as Coconut Island. These localities were selected as being representative of all types of intertidal habitats found on Oahu.

B. Distribution and abundance across the Coconut Island Reef: Seven Stations A through G were selected along a straight line transect from the shore (Station A) to the reef edge (Station G). The density of *Pomatoleios* was estimated from direct counts of tubes at each station. Separate estimates were made for the eastern (facing flood tidal current) and western (facing ebb tidal current) sides of each station. Two inch I beam steel stakes at Stations C, F, and G provided suitable substrate extending beyond the normal vertical range of *Pomatoleios* in both directions. At Station A the cement wall extended above this range only. The available surfaces on coral boulders at Stations B, D, and E were entirely within this range. At the same time, the abundance of the barnacle *Balanus hawaiiensis* Brock; the oysters *Ostrea sandvichensis* Sowerby and *O. gigas* (Thunberg); vermetid molluscs, the ascidians *Didemnun candidum* Savigny, *Tridideninun profundum* (Sluiter), *Symplegma* sp., *Botrilloides* sp.; and algae were recorded at all stations. The initial survey was made between June 24 and 28 and the final survey was made on October 21.

Larval settlement

Fouling plates (80 × 100 mm) mounted vertically at intervals of 1.5 inches, 12 to a frame, were placed at an inshore Station C and reef edge Station G two or 14 days before predicted larval settlement. (For details of the frame structure see Straughan, 1967b). The following types of plates and corresponding surfaces were mounted in two duplicated series per frame.

Clear glass		smooth, light, transparent
Ground glass		rough, dark, transparent
Black smooth glass		smooth, dark, transparent
Black smooth glass		rough, dark, transparent
Black bakelite (used as standard Plate)		smooth, dark, opaque
Asbestos cement	Side 1	smooth, light, opaque
	Side 2	pitted, light, opaque

Standard black bakelite plates mounted at an angle of 60° to horizontal were placed at both Stations two days before predicted settlement.

At Station C, 12 standard black bakelite plates were mounted horizontally 1.5 inches apart with the uppermost plate 11 inches above datum. This series was examined every 14 days from July 9 to October 25. On October 1 a second series of 12 plates was added 1.5 inches above the original series.

Spacing within populations of different densities was examined using distance to nearest neighbor as a measure of spatial relationships after the method of Clark and Evans (1954). The ratio (R) of observed mean distance between points of a population to the expected mean distance between points of a randomly distributed population serves as a measure of departure from randomness. In a random distribution, $R = 1$. Under conditions of maximum aggregation $R = 0$. Under conditions of maximum spacing $R = 2.1491$.

Survival

At low salinities: 50 adult specimens of *Pomatoleios* were placed in 2 gallon aquaria containing aerated water of salinities of 0‰ and 31‰ for varying periods before being returned gradually to seawater. The number of surviving animals was counted after 1 hour in seawater (salinity 35.5‰).

Under conditions of sand accumulation: 16 equal populations of established juvenile *Pomatoleios* were placed so that half were in positions free of sand and half in positions of sand accumulation. Survival in both series was determined after 14 days.

At high intertidal levels: In July established populations of 65 and 63 adult *Pomatoleios* were placed above the level of the *Pomatoleios* zone at Stations G (reef edge) and C (inshore) respectively, so that they were above the upper limit of settlement for that month but within the range of October settlement. Equal populations of adult *Pomatoleios* within the settlement range for July were used as controls. In all cases 50% of the population was on upper surfaces.

With "competition for space": The Spearman rank correlation coefficient

(Seigel, 1956) was used to show any association between the relative abundances of compound ascidians and *Pomatoleios*. The percentage of each fouling plate covered by compound ascidians per 14 days was compared with the survival of *Pomatoleios* on that surface.

Results and Observations

Distribution and abundance of Pomatoleios

A. Distribution around the coast of Oahu: Although many of the 12 species of the sub-family Serpulinae recorded from Oahu, were widely distributed around the island, *Pomatoleios* was found only in sheltered areas within Kaneohe Bay. The other localities visited were on the open coast and exposed to wave and surf action. *Pomatoleios* may also occur in Pearl Harbor where sheltered conditions similar to those in Kaneohe Bay exist. This area was not surveyed because of United States Navy restrictions. However, the high level of pollution in the Harbor may exclude *Pomatoleios*.

B. Distribution and abundance across the Coconut Island Reef in Kaneohe Bay: The following physical factors might contribute to limiting distribution and

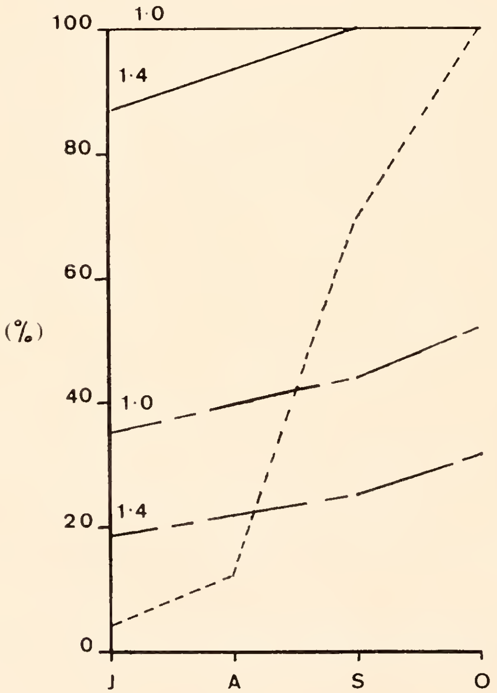

FIGURE 1. ———— = predicted percentage of days that 1.0 and 1.4 foot levels were submerged for part of the day; — ——— — = predicted percentage of hours that each level was submerged; — — — — = percentage of *Pomatoleios* settling on top 4 plates (= *Pomatoleios* zone) of the original 12 plate series, from July to October inclusive. Predictions were made from Tide Tables.

abundance of *Pomatoleios;* 1. Frequency and period of submergence, 2. Water movement, 3. Salinity, and 4. Temperature.

If other effects are ignored, the frequency and duration of submergence of a given level above datum can be predicted from tide tables. There is a gradual increase in the predicted frequency of submergence of the 1.0 foot and 1.4 foot levels from July to October (Figure 1). In July, the level of minimum High Water Spring (Min.H.W.S.) (that is, the highest level that is submerged every day) is 1.0 feet, while in September and October it is 1.4 feet. That is, in October, the predicted frequency that the 1.4 foot level is submerged equals that predicted for the 1.0 level in July, *i.e.*, once a day.

In July, the 1.0 foot level should be submerged 35% of the time, while in October, the 1.4 foot level should be submerged 31% of the time. On calm days during October, the tide remained above the predicted level although the tides approximated closely the predicted level in July. The rise in effective sea level was sufficient to increase the predicted time of submergence of 1.4 foot level in October to approximately that of the 1.0 foot level in July. This change most markedly affected larval settlement but also was responsible for changes in abundance.

Tidal current velocities inshore were 0.083–0.125 meters per second east to west on the flood tide, and 0.0415 meters per second west to east on the ebb tide. Current velocities on the reef flat were 0.25–0.3 meters per second east to west on flood tide and 0.09 meters per second west to east on the ebb tide. Therefore reef flat stations were exposed to currents of 2 to 3 times the velocity of those at inshore stations. Since the inshore stations are more sheltered from the north-northeast Trade Winds, the expected difference in current velocities would be greater during periods of trade wind influence. The eastward side of objects is always exposed to currents moving at a higher velocity than is the westward side.

Salinity remained close to that of normal seawater (35–36‰) throughout the survey except following heavy rain on September 1 and October 1. On each occasion salinity dropped to 31.5‰ on the surface at Station C for no longer than 24 hours. No resulting mortality was observed and experiments showed that adult specimens of *Pomatoleios* can survive at least 17 hours in freshwater (0‰) and 30 hours at a salinity of 31‰.

Temperatures were recorded on both the eastern and western sides of inshore Station C and reef edge Station G. A temperature range of 23 to 30° was recorded within the intertidal range of *Pomatoleios* at all sites. Therefore, animals exposed to the afternoon sun (western side) were subjected to similar upper temperature extremes as those exposed to the morning sun (eastern side). Inshore Station C is possibly exposed to higher water temperatures than the reef edge Station G because water flows over shallow reef flat areas before reaching C, while it flows from deeper areas to G.

While *Pomatoleios* was recorded at each of Stations A to G in June, the population was more abundant at Station C (12.0/sq inch) than at the other stations (Table I). At Station C, it occurred from 4 inches below datum to 11 inches above datum and at the top of this range formed a zone 4 to 5 inches wide.

Pomatoleios occurred up to a higher intertidal level and was more abundant at inshore Stations (A and C) than at reef flat or reef edge Stations (F and G) (Table I). At Station C, *Pomatoleios* extends to a higher level on the western side (11 inches above datum) which is exposed to low velocity water currents, than the eastern side (9.5 inches above datum) which is exposed to high velocity water currents. At Station B, *Pomatoleios* was more abundant on surfaces

TABLE I

Details of distribution and abundance of Pomatoleios *and general distribution and abundance of other organisms at Stations A to G*

Station	Range in inches	Individuals per square inch	Algae	*Balanus*	*Ostrea*	Vermetid	Ascidian
Station A (concrete wall)							
SIP	−2.0 to 15.0	2.5					
Station B (rusting drum)							
EIP	−4.0 to 1.5	0.625	XX				
WIP	−4.0 to 4.5	0.925	X				
EIP	−2.0 to 3.5*	0.725	XX				
WIP	−1.0 to 4.0	1.0	X				
Station C (iron stake)							
EIP	above 9.5	0.0		X	X		
	5.5 to 9.5	12.0		X			
	0.0 to 5.5	0.24	X	X	X	X	
	−4.0 to 0.0	0.125	XX				
WIP	above 11.0	0.0		X	X		
	6.0 to 11.0	7.0		X			
	0.0 to 6.0	0.24	X		X	X	X
	−4.0 to 0.0	0.125	XX				
Station D (dead coral boulder)							
EFU	0.0 to 5.0	0.375			X		
WFL	0.0 to 5.0	0.25	XX				
Station E (dead coral boulder)							
EFL	0.0 to 3.0	0.75		X		X	
EFU	0.0 to 3.0	0.5		X		X	
WFU	0.0 to 3.0	0.25	X	X		X	
Station F (iron stake)							
EFP	above 5.0	0.0		XX			
	2.0 to 5.0	0.5		XX			
WFP	above 5.0	0.0		XX			
	2.0 to 5.0	0.25	XX				
Station G (iron stake)							
ERP	above 6.5	0.0		X	X		
	−2.0 to 6.5	0.06		XX			
WRP	above 6.5	0.0		X	X		
	−2.0 to 6.5	0.0		XX			

S—surface facing south
E—surface facing east
W—surface facing west
I—inshore station
F—reef flat station
R—reef edge station
U—upper surface
P—perpendicular surface
L—lower surface
X—present on surface
XX—dominant on surface
*—sheltered by other side of drum

sheltered from water currents than on those exposed to water currents. That is, *Pomatoleios* is most abundant and extends to the highest intertidal levels at sites that are exposed to low water currents.

At Station A, *Pomatoleios* occurred from 2 inches below datum to 10 inches above datum on smooth surfaces, and to 15 inches above datum on creviced surfaces. Further at Station D, a coral boulder entirely within the *Pomatoleios* range, *Pomatoleios* occurred in crevices and not on outer surfaces. In the latter case, shelter from sand abrasion as well as shelter from water currents is probably a limiting factor.

At E where the boulder had relatively flat surfaces, in the absence of sand, *Pomatoleios* was more abundant on a lower surface than on an upper surface. Both surfaces faced the same direction and bore similar densities of other species. On the western surface where sand as well as algae occurred, *Pomatoleios* was less abundant than on the eastern surface.

At Station A, although *Pomatoleios* was not abundant (density 2.5 per sq inch), it was the dominant sedentary species throughout its range. At Station C below the *Pomatoleios* zone but above datum, algae, *Balanus, Ostrea,* vermetids, and compound ascidians were common, while below datum, algae was abundant. There is an increase in *Balanus* abundance from the inshore Station C to the reef flat Station F and the reef edge Station G. At Stations B, C, D, E, F, the density of *Pomatoleios* decreases where the algal abundance increases. Therefore, water currents, sand, and algae probably effect the distribution and abundance of *Pomatoleios.*

In October, the only change in the distribution of *Pomatoleios* was its presence (density = 0.25 per sq inch) in a band 5 inches wide above the *Pomatoleios* zone at Station C. At Stations C, F, G, compound ascidians were abundant up to 5 inches above datum while algae (predominantly a species of *Ulva*) formed a zone 5 inches wide above this. The abundance of algae at Stations B, D, and E also had increased. Hence at C the compound ascidians now extended to the bottom of the *Pomatoleios* zone while the algae extended over the *Pomatoleios* zone. Therefore, the rise in effective sea level between July and October, is reflected in the rise in the intertidal distribution of *Pomatoleios,* compound ascidians, and algae.

Larval settlement

Pomatoleios larvae settled during periods of spring tides throughout the study. Larval settlement was abundant during July, almost ceased during August and increased again during September and October. Unpublished data from other areas indicates that breeding ceases during the winter months at water temperatures of 23° C and below. There was a green algal bloom in the study area co-incident with the August reduction in larval settlement. At this time, few *Pomatoleios* adults in the study area contained mature genital products, while those kept in the laboratory contained mature genital products.

Sand accumulated rapidly and algae grew quickly on newly exposed surfaces. Therefore, to compare larval settlement on different types of surfaces, the surfaces were exposed within a few days of predicted larval settlement while to study the effects of sand and/or algae on larval settlement, surfaces were exposed 14 days before predicted larval settlement.

Larval settlement of *Pomatoleios* on horizontal fouling plates (Station C, July 9 to August 6) is shown in Figure 2. Larval settlement was more abundant below the *Pomatoleios* zone (= top four plates of July) than within this zone and extended below the lowest level of living *Pomatoleios.* In October, larvae settled on plates from 0.5 to 1.4 feet above datum. That is, in July the larval settlement range extended below the adult survival range while in October the larval settlement range extended above the adult survival range. This rise in larval settlement range parallels the rise in effective sea level between July and October.

Figure 1 shows that there was a gradual increase in the percentage of larvae settling within the *Pomatoleios* zone from July to October. The biggest increase in percentage of larvae settling within the *Pomatoleios* zone occurred in September. In September, the percentage of days that the *Pomatoleios* zone was submerged, rose to 100%. This suggests that frequency of submergence is more important than

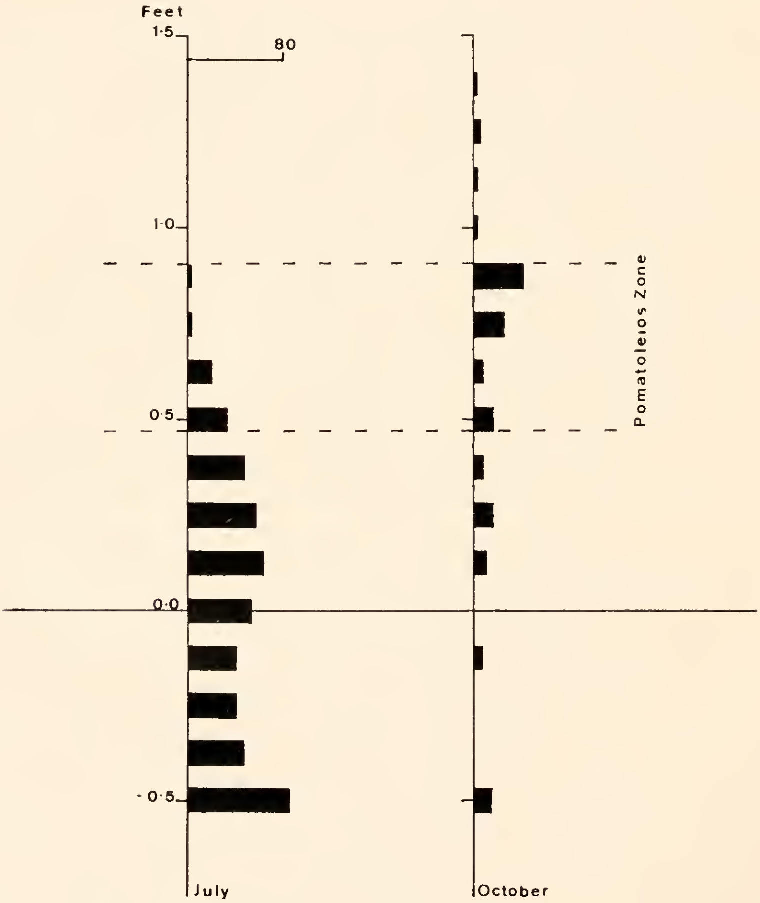

FIGURE 2. *Pomatoleios* settlement on 12 plates placed at the heights indicated during July. Number *Pomatoleios* on the above plates at the end of October and settlement on fouling plates placed above them during October.

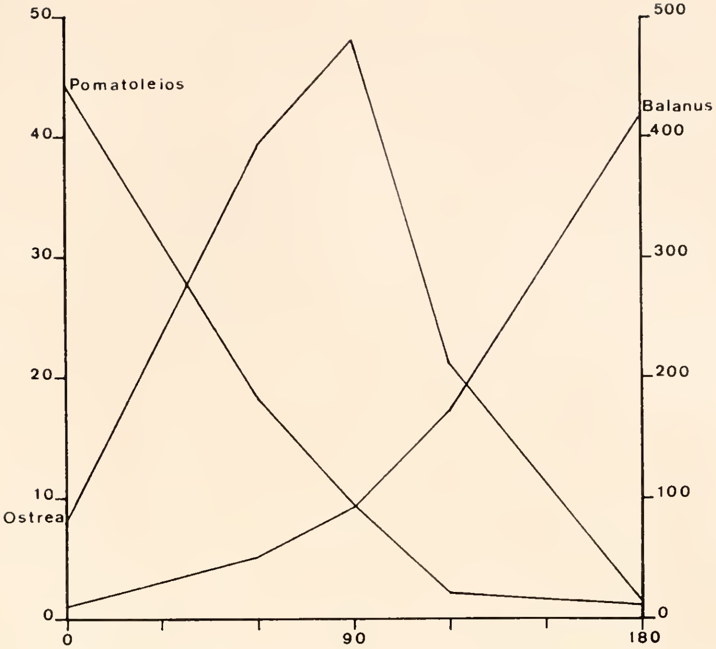

FIGURE 3. Number of larvae settling on fouling plates exposed at different angles. (0°–under surface; 90°–perpendicular; 180°–upper surface). *Pomatoleios* and *Ostrea* are plotted on the lower scale and *Balanus* on the higher scale.

overall percentage of time submerged in limiting the upper level of larval settlement.

Pomatoleios larvae did not settle evenly over the surface of fouling plates. In low density populations (density = 0.35 ± 0.03 per sq cm), individuals were randomly spaced (R = 0.93;0.88), while in higher density populations (density 0.6 per sq cm), they were aggregated (R = 0.417).

At Station A, larval settlement extended to a higher level on rough concrete than on smooth concrete. At Station C, experiments using fouling plates with different types of surfaces showed that larval settlement was also more abundant on a rough surface than on a smooth surface, and on a dark surface than on a light surface (Table II). Settlement on an evenly pitted surface did not differ from

TABLE II

Density (x per sq cm) Pomatoleios *settlement on different types of surface*

	Light transparent	Light opaque	Dark transparent	Dark opaque
Smooth	0.125–0.25	0.375	0.375	1.125–1.625
Evenly pitted		0.375		
Rough	0.5		2.0	

settlement on a smooth surface. Of the surfaces tested, larvae settled most abundantly on a dark, rough, transparent surface. However, this type of surface was not compared with a dark, rough, opaque surface which is probably even more suitable for larval settlement.

In Figure 3, larval settlement on fouling plates exposed at different angles at the same height above datum, is compared. *Pomatoleios* settlement was most abundant on the underside of objects (0°), and decreased with increasing angle until hardly any settlement occurred on the upper surface of objects. Of the other common intertidal species that may compete with *Pomatoleios, Balanus* larvae settled most abundantly on the upper surfaces (180°) and settlement decreased with decreasing surface angle. *Ostrea* larvae settled most abundantly on vertical surfaces but settlement was more abundant on the lower than the upper surfaces.

TABLE III

Number of larvae settling on fouling plates

Species	Reef edge		Inshore	
	Exposed	Sheltered	Exposed	Sheltered
Pomatoleios	0	0	0	12
	0	0	0	9
	0	0	0	13
	0	0	0	14
Balanus	56	25	0	0
	105	77	0	0
	104	48	8	0
	60	17	0	0
Ostrea	43	20	8	0
	84	46	0	0
	21	15	5	0
	16	9	0	0

Comparison of settlement of *Pomatoleios, Balanus,* and *Ostrea* on vertical fouling plates under conditions exposed to and sheltered from water currents at Station C (inshore) and Station G (reef edge) is made in Table III. *Pomatoleios* larvae settled more abundantly on inshore than reef edge fouling plates, and more abundantly on sheltered than exposed surfaces. In contrast, *Balanus* and *Ostrea* larvae settled more abundantly on reef edge than inshore fouling plates, and more abundantly on exposed than on sheltered surfaces. Therefore, there appears to be very little, if any, competition for space during larval settlement between *Pomatoleios* and *Balanus* and *Ostrea* larvae.

It is difficult to separate the effect of some physical factors on larval settlement, for example, current velocity and sand, and current velocity and presence of an algal mat. The latter at two weeks is composed of short algal species which trap some sand, and has a maximum thickness of 0.5 mm. In Table IV, the density of larval settlement under these varying conditions is tabulated. Situations where algae was absent and where sand was common, were always sheltered from the current, that is, alternatives "a" and "a_1" did not exist. No settlement occurred in high current velocity conditions, while under low velocity conditions, settlement was more abundant in the absence than presence of sand and the algal mat.

Survival

It was noted in the initial survey in July, that the adult population is more abundant in the absence than presence of sand and/or algae, and (above) that larval settlement is similarly effected. When sand accumulated after larval settlement a 25% mortality was recorded in six weeks. However, when established juveniles that settled in positions sheltered from sand, were placed in positions where sand accumulated, the mortality in two weeks was greater than that recorded among specimens that settled on the sandy side. Mortality also increased with the increasing accumulation of sand. (Thin layer of sand—25–37% mortality; 0.5 mm sand layer—34% mortality; 1.0 mm sand layer—56% mortality.)

Table IV

Density (x per sq cm) Pomatoleios *settlement on fouling plates exposed to currents of high and low velocities, in presence and absence of sand and algal mat*

Current	Sand		Algal mat	
	Absent	Present*	Absent	Present
High velocity	0.0	a	a_1	0.0
Low velocity	1.625	0.25	1.25–3.75	0.0

* 0.5 mm thick in 2 weeks.
Conditions represented by a, a_1 did not exist.

While the upper limit of larval settlement in July was the top of the *Pomatoleios* zone, it was possible that the more tolerant adults could survive at a higher intertidal level. However, when specimens were placed above the *Pomatoleios* range, at the reef edge (Station G), all (65) were destroyed by crabs in four weeks. Inshore (Station C), crabs destroyed 20% of the specimens above the *Pomatoleios* range but none within the *Pomatoleios* range. At the higher level, none of the remaining tubes on the upper surfaces contained living specimens of *Pomatoleios,* while 50% of the tubes on the lower surfaces contained living specimens of *Pomatoleios.* There was a 95% survival of specimens on upper and lower surfaces within the *Pomatoleios* zone. At these mortality rates, *Pomatoleios* could not survive above the *Pomatoleios* zone over the summer months. That is, larvae settling above the *Pomatoleios* zone at C during October, could not survive the following summer.

Allan Miller in a study of the feeding habits of *Morula,* found that *M. granulata* will prey on *Pomatoleios* (personal communication). However, *M. granulata* is most abundant in the upper intertidal areas on the exposed coast and it was not collected from the sheltered inshore areas where *Pomatoleios* was most abundant. Hence predation by *M. granulata* was probably marginal.

Although *Balanus* and *Ostrea* larvae settle least abundantly where *Pomatoleios* settle most abundantly, they, as well as vermetids, possibly compete for space with *Pomatoleios* after settlement. While *Balanus* attained a density of up to 1/10 sq mm in four weeks, it did not cause any observable mortality within the *Pomatoleios* population. *Ostrea* and vermetids were less abundant than *Balanus* and co-existed with *Pomatoleios* in the marginal areas of the *Pomatoleios* range. Following the green algal bloom in early August, *Balanus* and *Ostrea* mortality was 75% while no *Pomatoleios* mortality occurred on fouling plates.

Several species of colonial ascidians grew rapidly in the subtidal and lower intertidal areas. The level at which colonial ascidians were common rose from 0.1 feet below datum in July, to 0.1 feet above datum in August, to 0.4 feet above datum in September, to 0.6 feet above datum in October on horizontal fouling plates. On vertical surfaces, colonial ascidians only extended to 0.4 feet above datum in October.

A comparison of the area covered by colonial ascidians and *Pomatoleios* survival, shows that *Pomatoleios* survival decreases as the area covered by colonial ascidians increases (Table V). Using the Spearman Rank Correlation Coefficient (Siegel, 1956), $r = -0.785$. This negative correlation is significant between 0.05 and 0.01 levels for a one tailed test. *Pomatoleios* mortality on the lower ten fouling plates (-0.5 to $+0.6$ feet) between July and the end of October (Fig. 3), was the result of competition from colonial ascidians.

TABLE V

Pomatoleios *survival with increasing ascidian competition per 2 weeks*

Area occupied by ascidian (%)	*Pomatoleios* survival (%)
0–10	95–100
10–20	70
20–30	60–65
30–40	50
40–50	65
50–60	35
60–70	55
70–80	40
80–90	10–20
90–100	0

As the effective sea level rose from July to October, the upper limit of the colonial ascidians rose and extended to the bottom of the *Pomatoleios* zone at C in October. Therefore during autumn, colonial ascidians compete for space with *Pomatoleios* that settled below the *Pomatoleios* zone during summer. Hence the lower level of the *Pomatoleios* zone at C was controlled by competition with colonial ascidians.

DISCUSSION

Pomatoleios forms a narrow well-defined zone in sheltered intertidal areas. However, its survival range extends subtidally and the larval settlement range extends intertidally above the survival range during autumn and subtidally below the survival range during summer.

Lewis (1964, p. 217) states "it nevertheless appears from comparisons of many shores that wave action is primarily responsible for raising zonal boundaries, especially those of the upper shore where it is a matter of raising the theoretical height of the sea." While he was referring to localities exposed to different amounts of wave action, the present species shows a seasonal change in larval settlement height due to seasonal influence of the North Pacific Swell, trade winds, and changes in the predicted submergence at different levels. In this case, where the maximum tidal range is 3.0 feet, a shift in tidal levels of 0.4 to 0.6 feet effects a large portion of the intertidal environment. *Pomatoleios* settlement range changed seasonally as did the position of Min.H.W.S. (position that is submerged at least once a day) which is higher during autumn and spring than during summer and winter. In spring and autumn, most larvae settle within the *Pomatoleios* zone, while in summer, settlement is most abundant below the *Pomatoleios* zone. Settlement ceases during winter.

While the *Pomatoleios* settlement zone is higher on the shoreline in October than July, so is the distribution of algae and ascidians. The ascidians extend to the bottom of the *Pomatoleios* zone during October and kill specimens of *Pomatoleios* that settled below the *Pomatoleios* zone in summer. Algae extend into the lower *Pomatoleios* zone but do not affect specimens of *Pomatoleios* already present. However, continuous algal movement and accumulation of sand reduce larval settlement. Larvae which settle above the *Pomatoleios* zone during spring and autumn are reduced in numbers during summer and winter. They are attacked by crabs, exposed to air for long periods when the level of Min.H.W.S. falls, and are exposed to low salinities.

Connell (1961, p. 722) states that "the lower limit of distribution of intertidal organisms is mainly determined by the action of such biotic factors as competition for space or predation. The upper limit is probably more often set by physical factors." In *Pomatoleios,* while exposure to physical factors was important in limiting the upper level of the population, predation by crabs was also an important factor. No predation was recorded at lower intertidal levels where the population appeared to be limited entirely by seasonal competition for space with colonial ascidians.

Lewis (1964, p. 230) states "It is important, however, to appreciate that although larval discrimination is an important and perhaps necessary adaptation to zonation, it does not explain zonation." However, larval discrimination during settlement may be more important in the Serpulinae than in other groups of zone forming species. *Mercierella enigmatica,* another zone forming serpulid, is also known to aggregate during larval settlement (Straughan, unpublished). Both these serpulid species are able to build their tubes in any direction so that intraspecific competition for space is not as important as in species where shape and size are less variable, for example, barnacles and spirorbids. At high population densities, the former form unstable communities that are easily dislodged by wave action, while the latter space themselves out during settlement (Knight-Jones and Moyse, 1961). Hence aggregation during settlement is probably more important in building up a zone in the Serpulinae than in groups where intraspecific competition for space occurs at high population densities; it reinforces the effect of differential survival of the adults.

Velocity of waterflow over the substrate, type of exposed surface (texture light, and color), and angle at which the surface is exposed, are factors effecting settlement at all depths. The fact that larvae did not differentiate between the surface with large even pits and a smooth surface, but showed a definite preference for a rough surface, suggests that the latter was preferred because it enabled firmer tube attachment.

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SUMMARY

Pomatoleios kraussii forms an intertidal zone which extends from 0.5 to 0.9 feet above datum in the sheltered areas of Kaneohe Bay. The settlement range which extends subtidally, is wider than the adult survival range which in turn is wider than the *Pomatoleios* zone. The settlement range moves up and down the shore corresponding to seasonal changes in the level of minimum High Water Springs. The *Pomatoleios* zone is limited at the top by exposure to air and predation, and at the bottom by competition for space. Habitat selection within the settlement zone is influenced by surface texture, surface angle, exposure to currents, presence of sand and algae. Competition for space with *Balanus, Ostrea,* and vermetids is largely eliminated by different larval settlement preferences in these species.

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ON THE TRYPSIN SENSITIVITY OF GAMETE CONTACT AT FERTILIZATION AS STUDIED WITH LIVING GAMETES IN CHLAMYDOMONAS[1]

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Specific sensitivity to enzymes has been a useful means for the characterization of sex cell interaction at fertilization by revealing the nature of the participating functional structures of the gamete surfaces (*cf.* Metz, 1954; *cf.* Runnström, Hagström and Perlmann, 1959; *cf.* Metz, 1967; Brock, 1965; Crandall and Brock, 1968; Taylor and Orton, 1967). Appropriate enzymes may be expected to interfere with one or more steps in the attachment and fusion processes at fertilization or to reduce its specificity. Such effects have been described in sea urchin fertilization. Trypsin treatment reduces the fertilizability of the eggs, probably by elimination of the sperm receptor substance(s) (Tyler and Metz, 1955; Runnström and Kriszat, 1960). Trypsin treatment of the eggs facilitates cross-fertilization in the sea urchin (Hultin, 1948ab; Tyler and Metz, 1955; Hagström, 1959) and eliminates self sterility in the hermaphroditic ascidian *Ciona* (Morgan, 1939; Bohus-Jensen, 1958). These effects of trypsin indicate that the components of the interacting systems at fertilization are proteinaceous or are closely associated (*i.e.,* anchored to) proteinaceous material. In addition, proteases provided the first evidence for the protein nature of isolated attachment substances (Tyler and Fox, 1940).

The isogametic copulation of the Chlorophycean, *Chlamydomonas,* provides a simple model-like example of sexual differentiation and of fertilization events for analysis. In the isogamous, heterothallic species used in these studies, gamete union proceeds in two steps (*cf.* Coleman, 1962; Wiese and Jones, 1963). The initial mating type reaction effects a specific agglutinative adhesion between gametes of opposite sex. This adhesion occurs at the flagella tips and implies the interaction of specific mating type substances (*cf.* Wiese, 1965). Subsequent to the mating type reaction the agglutinated gametes unite into pairs: In *C. moewusii* and *C. eugametos* two sexually different gametes establish papillar contact at the basis of their flagella. The papillae fuse and form a protoplasmic bridge which connects the two cells resulting in a peculiar prezygotic stage, the *vis-à-vis* pair. After papillar attachment the agglutinated flagella separate, and the *vis-à-vis* pair moves and behaves as one physiological unit. Finally, after 18–36 hours, the two gametes fuse completely and caryogamy occurs. The mating type substances have been isolated and act as isoagglutinins (*cf.* Wiese, 1965): each component makes gametes of its respectively opposite sex agglutinate one with another.

These isoagglutinations are assumed to result from bi- or multivalency of the isoagglutinins, in keeping with immunological doctrine. Trypsin and pronase have been used to examine the functional structure and chemical composition of the isolated mating type substances (Wiese and Wiese, in preparation). This paper deals with the effects of trypsin on the mating type substances *in situ* and on the entire copulation process. Trypsin was selected because its pH-optimum coincides with that for mating in *Chlamydomonas* (Wiese, unpublished) and because the mode of its proteolytic action is known (Bergmann and Fruton, 1941).

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MATERIAL AND METHODS

The experiments were performed with *Chlamydomonas eugametos* and with *C. moewusii* syngen I which has been identified by complete sexual compatibility with the Indiana strains Collection No. 96/97 (*cf.* Starr, 1964). The species specificity of the mating type reaction during the trypsin treatment was checked with gametes of *C. reinhardti, C. mexicana*, and *C. moewusii* syngen II (Indiana Collection No. 792/793, sexually incompatible with No. 96/97 and with *C. eugametos*). These two strains were earlier designated as *Chlamydomonas* spec. (cf. Wiese, 1965). We label No. 793 as the (−) strain and No. 792 as the (+) strain since the latter's gametes are responsible for the locomotion of the *vis-à-vis* pair. By this feature strain No. 792 corresponds to the (+) sex of *C. moewusii* syngen I (*cf.* Lewin, 1952) and to the male sex of *C. eugametos* (*cf.* Wiese, 1965).

All strains including *C. reinhardti* were cultured on KNOP-agar (Wiese, 1965) at 19 ± 1° C and with an illumination of 1200 Lux given in a light cycle of 16^h light and 8^h dark. [*C. reinhardti* grows excellently on nitrate as sole nitrogen source.]

The vegetative cells were induced to undergo gametogenesis by flooding the agar slants overnight with sterile 0.02 *M* TRIS-buffer, pH 7.6. In the morning the gametes were washed by centrifugation at 180 g for 5 minutes and resuspension in the TRIS-buffer containing 0.01 g/l $CaCl_2$, 0.01 g/l K_2HPO_4, and the respective $MgSO_4$-concentration applied, *i.e.*, 0.025 or 0.0125%. The cell density of the suspensions, as determined by hematocrits, was always adjusted for 4 cubic millimeters of packed cells per milliliter.

Salt free, 2 x crystallized trypsin (Worthington Biochemical Corp., Freehold, New Jersey) and 5 x crystallized soybean inhibitor (Nutritional Biochemical Corp., Cleveland, Ohio) were used in the experiments.

With respect to the mating type reaction, the degree of the sexual reactivity of treated and control gametes was assessed microscopically and scaled from − (no agglutination) to + + + (agglutination of virtually all gametes). Since the mating type reaction is an obligatory prerequisite to pairing, the intensity of the mating type reaction can also be determined quantitatively by the ratio of paired to unpaired cells provided that the trypsin influence upon pairing, as described in this paper, is eliminated by application of trypsin-inhibitor. On the other hand, when the mating type reaction is not affected the number of pairs obtained in experimental and control mixtures provides a selective measure of the inhibition of pair formation. Additional details are given in the text.

RESULTS

Trypsin exerts a distinct inhibitory action on fertilization of *Chlamydomonas moewusii* and *C. eugametos*. By varying trypsin concentrations and manner of application it was possible to show that both steps in fertilization, namely the mating type reaction and the pairing, are trypsin-sensitive. Both events can be blocked selectively. The "vegetative" functions of the *Chlamydomonas*-gametes including those prerequisites for copulation such as flagellation and motility, are not visibly affected by the trypsin treatments employed. Gametes show undiminished motility even after 24 hours at 26° C in 0.1% trypsin, the strongest solution used.

The trypsin-sensitivity of the pairing

A selective action of trypsin on this second phase of the copulation process can only be demonstrated if the trypsin treatment leaves the initial agglutinative mating type reaction unaffected. In preliminary experiments pairing was found to be markedly reduced in 0.025% trypsin solutions, whereas the capacity of the flagella tip to agglutinate was not affected by this trypsin concentration. Gynogametes and androgametes which have been pretreated with this concentration for two hours agglutinate immediately and quantitatively upon mixing. Therefore, 0.025% trypsin was used to investigate the trypsin sensitivity of the pairing.

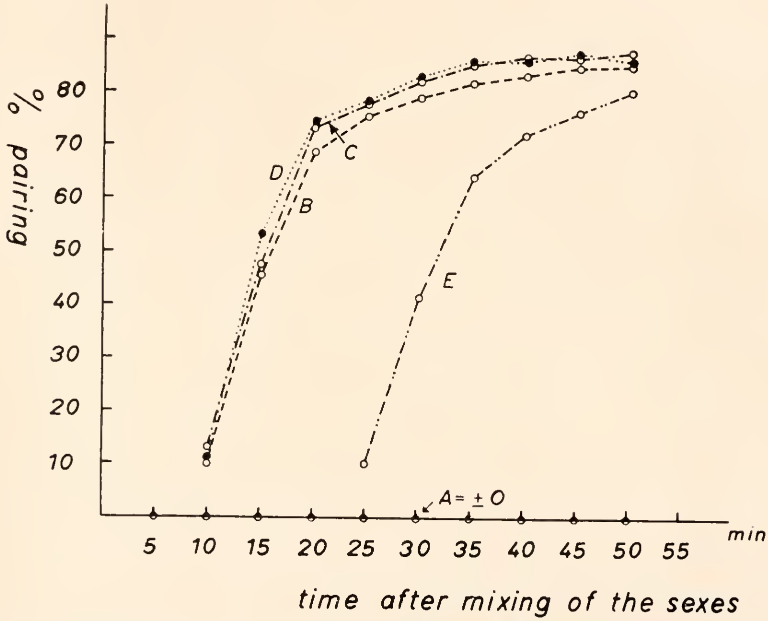

FIGURE 1. Trypsin inhibition of the pairing in *C. moewusii* (for detailed information see text). A. Complete inhibition by 0.025% trypsin. B. Trypsin effect entirely counteracted by 0.05% trypsin-inhibitor. C. Pair formation in the buffer control. D. Failure of 0.05% trypsin-inhibitor to affect pairing. E. Approximately uninhibited pairing in 0.025% trypsin after delayed application of 0.05% trypsin-inhibitor.

The selective action of this trypsin concentration upon pairing in *C. moewusii* syngen 1 is shown in Figure 1. Gamete suspensions of both sexes were prepared overnight as outlined in the methods sections. In the morning the two gamete types were mixed and immediately 50 ml of the mixed suspension were added to each of the following samples:

A. 50 ml Tris-buffer + 25 mg trypsin + 6.25 mg $MgSO_4$
B. 50 ml Tris-buffer + 25 mg trypsin + 6.25 mg $MgSO_4$ + 50 mg trypsin inhibitor
C. 50 ml Tris-buffer + 6.25 mg $MgSO_4$
D. 50 ml Tris-buffer + 6.25 mg $MgSO_4$ + 50 mg trypsin inhibitor
E. 50 ml Tris-buffer + 25 mg trypsin + 6.25 mg $MgSO_4$. 50 mg trypsin inhibitor (dissolved in additional 10 ml buffer) were added to this sample after 15 min.

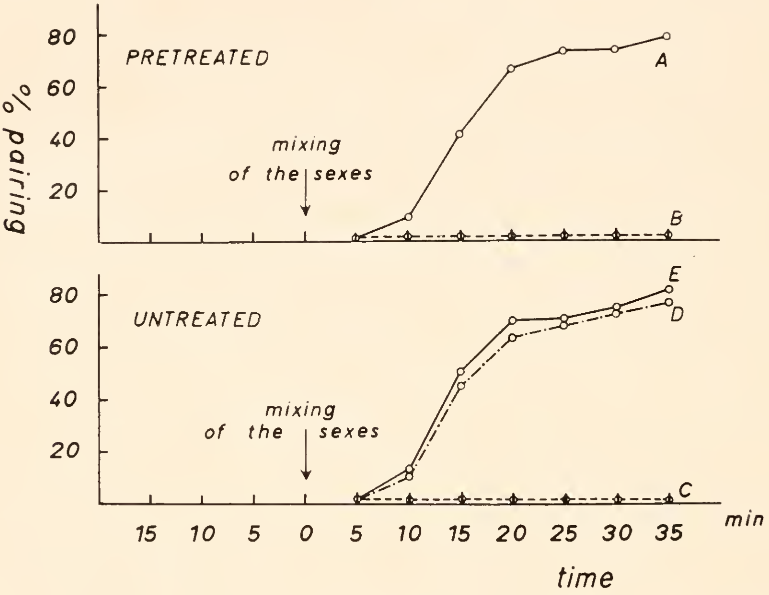

FIGURE 2. Trypsin inhibition of pair formation in *C. moewusii*. Different application of trypsin (at mixing of the sexes or as pre-incubation) in presence or absence or trypsin inhibitor. A and B: Gametes pretreated with 0.025% trypsin 15 min. before mixing of the sexes. A. Trypsin inhibitor (0.05%) added at mixing; trypsin content adjusted to 0.025% final concentration. B. Without inhibitor. C, D and E: Same material not pretreated. Added at mixing of the sexes were C. 0.025% trypsin, D. 0.025% trypsin and 0.05% inhibitor, and E. 0.05% inhibitor.

Microscopic examination of the samples showed that during the entire course of the experiment the trypsin-treated gametes were not impaired with respect to flagellation, motility, and agglutinability. However, the trypsin-treated samples showed markedly reduced pair formation. In a representative experiment (Fig. 1) the control gamete mixture yielded over 80% pairing (curve C 87.2%, curve D 86.3%) whereas the trypsin-treated sample (curve A) was practically devoid of pairs. Trypsin-inhibitor, added simultaneously with trypsin at the mixing of the gametes, yielded full pairing (curve B, 85.6%). Addition of trypsin-inhibitor 15 minutes after trypsin application still yielded 80.6% pairing (curve E).

The data of Figure 1 clearly show that treatment with trypsin in the appropriate concentration selectively inhibits pair formation. This effect is assumed to result from the proteolytic action of the enzyme since the effect is not obtained in the presence of trypsin-inhibitor.

The failure to pair could be due to an enzymatic interference with the process of pair formation or to the enzymatic elimination of functional components at the prospective attachment sites. Such functional components essential for papillar attachment and adhesion could exist either before the mating type reaction, or could be formed or set into action only after the mating type reaction has ensued.

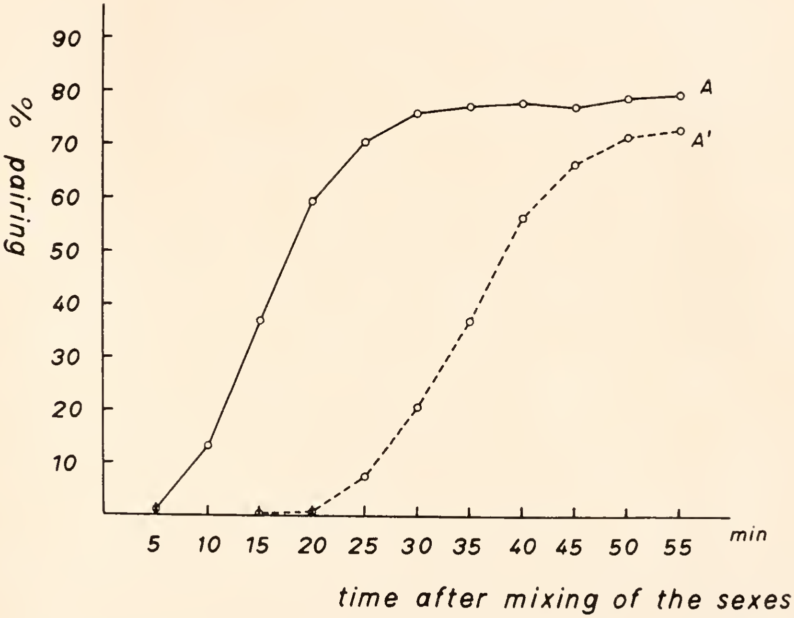

FIGURE 3. Trypsin-sensitive phase of pair formation in *C. moewusii*. After mixing of both gamete types samples were taken at 5 min intervals, incubated with 0.025% trypsin for 15 min and fixed afterwards (A′-data). Compared with the normal formation rate of pairs (Curve A) each A′ value has to be related to the percentage of pairs originally present in the sample in question as well as to the percentage to which the pairing has meanwhile proceeded.

According to Figure 1 (curves A and C) trypsin prevents any pairing when added simultaneously with the mixing of the two gamete types. About 5–7 min elapse between the initial agglutination and the appearance of free-swimming and fixation-resistent pairs. If pairing is prevented by the enzymatic elimination of preexisting attachment sites, the 5–7 minute time lapse would have to be sufficient to eliminate the sites. However, gametes which were pretreated with trypsin for twice that length of time, did not show any inhibition of the pairing if sufficient trypsin-inhibitor was added at the time of mixing of the sexes (Fig. 2). Evidently, inhibition of the pairing by trypsin does not result from the enzymatic destruction of preexisting components at the gametes' apices. This fact is also indicated by the appearance of pairs after delayed addition of trypsin inhibitor (Fig. 1, E). The inhibitory effect is exerted during the formation of the plasmatic bridge.

To further explore this trypsin action, the effect of short duration treatments throughout the copulation process was investigated. A (+) and a (−) gamete suspension were mixed. At 5 minutes intervals two samples of one ml volume were taken from the mixture. One sample (A-series) was diluted with one ml TRIS-buffer containing 0.0125% $MgSO_4$ and immediately fixed by addition of 2 drops of aqueous Lugol's solution. To the other sample one ml of the buffer containing 0.05% trypsin and 0.0125% $MgSO_4$ was added and this sample (A′ series) was fixed after 15 minutes. Such "pulse treatment" with trypsin reveals when the trypsin-sensitive phase begins, and detects its temporal relationship to the final fusion of the gametes (Fig. 3). From the curves A and A′ it is evident that (1) trypsin stopped the pairing; (2) trypsin is able to split to a considerable degree

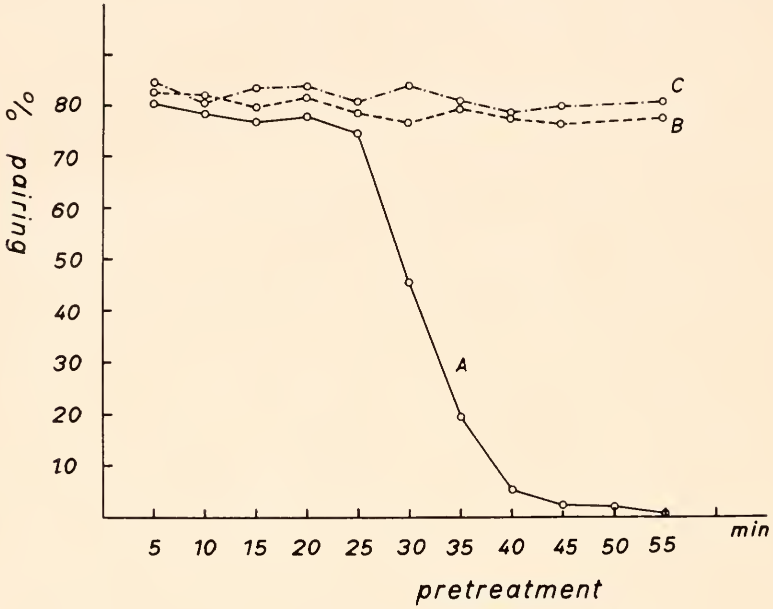

FIGURE 4. Trypsin sensitivity of the mating type reaction in *C. moewusii* as determined by the pair test. A. Inactivation of the (−) gametes. 150 ml (−) gamete suspension (8 mm^3 packed cells/ml) were incubated with 0.1% trypsin. At 5 minute intervals, 5 ml samples were taken and the incubation was interrupted by adding 5 ml 0.1% trypsin-inhibitor. After addition of 10 ml untreated (+) gametes (4 mm^3 packed cells/ml), the number of pairs was stated fixing each sample after 30 minutes. B. Trypsin effect upon the (+) gametes checked correspondingly with untreated (−) gametes. C. Control indicating the virtually unaltered agglutinating capacity of both gamete suspensions during the time of the experiment. 5 ml of both untreated gamete types (8 mm^3 packed cells/ml) were combined in 5 minute intervals and 10 ml 0.05% trypsin-inhibitor solution were added. The percentage of pairs formed in each sample within 30 minutes is determined.

pairs which had been so well established that they resist fixation (in the corresponding A-sample); and (3) the ability to split pairs declines with the length of time the pairs have been established until finally the papilla fusion proceeds to a condition no longer divisible by trypsin. The special trypsin-sensitive period exists only at the beginning of the pairing stage.

In all these points gametes of *C. eugametos* gave similar results when subjected to the same experimental procedures.

The trypsin-sensitivity of the mating type reaction

The experiments reported in the previous section were conducted on the premise that trypsin, in the concentrations and exposure times used, had little if any effect on the initial mating type reaction. At higher trypsin concentrations, however, the flagellar agglutination, too, was affected in a specific manner. This particular effect was best demonstrated with a concentration of 0.1% trypsin which did not interfere with the flagellation or the locomotion of the gametes. The Mg^{++}-concentration was kept at 0.025% in order to prevent any ionic disturbance of the agglutination process. This trypsin action, too, can be prevented by addition of trypsin-inhibitor.

In a mixture of androgametes and gynogametes incubated with 0.1% trypsin at 26° C for a longer period, the initial intensive agglutination of the gametes gradually decreases and is finally, after 45–60 minutes, lost entirely. No pairs are formed. Separate pre-incubation of the two gamete types and mutual checking with untreated test gametes revealed for *C. moewusii* syngen I that it is exclusively the (−) sex which is sensitive (Fig. 4). Thus the trypsin pretreated (+) or androgametes agglutinated strongly with test (−) gametes whereas trypsin pretreated gynogametes failed to agglutinate with (+) test gametes.

The different effects exerted on the mating type reaction and on pairing permitted the use of the pair test as a quantitative measurement for the inhibition of the mating type reaction, provided that the action of trypsin on pairing was neutralized by trypsin-inhibitor added at the time of mixing of the gametes (Fig. 4).

Again, *C. eugametos* reacts in an entirely similar manner; one mating type is sensitive and the other is resistent to trypsin. The trypsin-sensitive type (female) corresponds to the (−) sex of *C. moewusii* syngen I.

Since the species specificity of copulation is associated with the mating type reaction (*cf.* Wiese, 1965), the conservation of this specificity was examined during the action of 0.1% trypsin. At 5 minutes intervals, samples of the 4 treated gamete types (*C. moewusii* syngen I (+) and (−), *C. eugametos* male and female) were combined with highly active gametes of *C. reinhardti*, *C. moewusii* syngen II, and *C. mexicana*. Any possible loss of specificity was tested by checking for the appearance of non-specific flagellar agglutination. Each sex of *C. eugametos* and *C. moewusii* syngen I was checked against both sexes of the incompatible forms; no case of a non-specific agglutination was observed.

DISCUSSION

Among the sequence of events proceeding at copulation, the two trypsin-sensitive steps namely the mating reaction and papillar union, are well defined and can be selectively influenced. Thus, a higher trypsin concentration and longer incubation time is required to inhibit the mating reaction than to inhibit pairing. Among the species that have been cross tested, the species specificity of copulation remains unaltered by treatment with trypsin.

The trypsin influence on the mating type reaction

The action of trypsin on the mating type reaction is characterized by its unilateral inhibition of the (−) sex alone. Likewise, in the sea urchin, the trypsin sensitivity of fertilization is unilateral and restricted to the egg whereas the fertilizing capacity of the sperm remains unaffected (Tyler and Metz, 1955; Hagström, 1959).

In *Chlamydomonas,* the unilateral or differential effect of trypsin can be explained in at least two different ways as follows: (1) the (−) mating substance but not the (+) substance is inactivated by trypsin. (2) Both the (+) and the (−) substances are inactivated by trypsin but the (+) substance is replenished continuously by a gametogenic metabolism specific to the (+) or androgamete (Förster and Wiese, 1954; Förster, 1957, 1959; Stifter, 1959; Hartmann, 1962). On the other hand the essential amount of (−) substance would be produced at gametogenesis by the gynogamete, and once destroyed by trypsin, no new (−) substance would be produced to the extent that the mating reactivity will be restored.

The fact that both isoagglutinins are sensitive to trypsin (Förster, Wiese and Braunitzer, 1956; Wiese, 1961; Wiese and Wiese, in preparation) does not decide in favour of the second alternative since the detached components (isoagglutinins) might well be more sensitive to tryptic attack than the same components *in situ.* Moreover, the capacity to produce isoagglutination requires a functional bi- or multivalent structure, whereas gamete adhesion presumably could result from interaction of univalent complementary cell surface substances. Accordingly, loss of the multivalent structure would destroy the agglutinating action of the substance in solution, but not necessarily the adhesive properties of the material at the cell surface.

Analyses of the effect of trypsin on the isoagglutinins and of the supernatants of trypsin-treated gametes are expected to answer this question. Evidence so far available indicates that a macromolecular component which is split off during the tryptic inactivation of the gynogametes, has no capacity to induce isoagglutination of the androgametes (Wiese and Wiese, in preparation). In the yeast, *Hansenula,* trypsin inactivates one mating type by splitting off its mating substance (Crandall and Brock, 1968). The mating substance of the other mating type can be detached by a snail enzyme preparation (Taylor, 1964) or by subtilisin (Taylor and Orton, 1967). In both mating types, the enzymatically detached components have here retained their capacity to combine with the complementary gametes.

A further possibility that the disappearance of the sexual activity on addition of trypsin is not a specific enzyme effect at all, but rather a differentiation back to the vegetative stage caused by the supply of a metabolizable N-component, is excluded by the non-appearance of the trypsin effect on addition of trypsin plus trypsin inhibitor. This possibility also seems highly improbable because addition of peptones, glycine, alanine, trypsin-inhibitor, and normal rabbit serum in no way interferes with the sexual activity (Wiese, unpublished). Such an interference complicated the corresponding analysis of the conjugation process in *Paramecium* (Metz and Butterfield, 1951). Trypsin incubation of dead gamonts which were killed without destroying their mating type activity, revealed a bilateral trypsin-sensitivity of the mating type reaction (*cf.* Metz, 1954). A difference between the mating types was demonstrated by means of nitrous acid and certain formalin concentrations. These inactivated the reactivity in one mating type only and offered a hint to the nature of the reactive groups involved (*cf.* Metz, 1954).

The influence of trypsin on pair formation

The specific elimination of pairing in an organism with a two-step gamete adhesion and fusion sequence was first demonstrated in *Chaetomorpha* after application of subtilisin (Köhler, 1956). It was concluded that the different enzyme sensitivities of pair formation and flagella agglutination, respectively, resulted from different chemical bases of the two steps. In *Chlamydomonas,* the two steps do result from two different mechanisms as shown by their different sensitivity to SH-reagents and to laurylsulfate (Wiese and Jones, 1963). This difference, however, cannot be derived directly from the different sensitivity to trypsin, since there are apparently no prospective attachment sites at the papillae comparable to the flagella agglutinin. Trypsin-pretreated gametes do pair when trypsin-inhibitor is added at the moment of mixing of the two gamete types. Addition of inhibitor after the mixing (Fig. 1, E) is as effective and even more informative in case such attachment sites would not be existent (or susceptible) before the final completion of the flagella attachment, *i.e.,* if the capacity to make papilla attachment would arise as an induction effect from the prior flagella attachment. The effect on pairing extends to an elimination of the morphogenetic process of the bridge formation in concentrations of 0.025% trypsin and higher. Slightly lower concentrations may permit bridge formation to proceed but render the established pairs so weakened that the pair mates separate on subsequent fixation. The trypsin-sensitive period extends beyond the stages at which the papilla attachment is achieved, the flagella attachment discontinued, and the decision made on the different behavior of the (+) and the (−) flagella. During this period, even pairs which are resistant to fixation may still be split by trypsin (Fig. 3). The mates of older pairs, however, are no longer separable.

SUMMARY

Trypsin affects the copulation of isogametes in *Chlamydomonas* (*Chlorophyceae*) in two ways. In low concentrations it rapidly suppresses pair formation by interfering with the formation of the protoplasmic bridge between the gynogamete and the androgamete. In the early phase of its formation the bridge may even be split by the action of trypsin. In higher concentrations, trypsin additionally affects the mating type reaction by destroying the capacity of the gynogametes to agglutinate with the androgametes. The agglutinating capacity of the androgametes is not impaired by trypsin. Trypsin does not affect the species specificity of the attachment mechanism within the system tested.

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ON THE ABSENCE OF CIRCADIAN RHYTHMICITY IN *DROSOPHILA PSEUDOOBSCURA* PUPAE

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Circadian rhythms are the rule rather than the exception in eucaryotic organisms; from unicellular organisms to higher plants and animals a diversity of physiological functions has been shown to exhibit approximately 24 hour intervals between maxima. In spite of this physiological and systematic ubiquity, the *circadian oscillations* underlying circadian rhythms are remarkably uniform in their formal properties: they persist in constant dark and different constant temperatures with a period, τ, which is close to 24 hours, and they can be reset, and entrained by light and temperature signals (for reviews, see Pittendrigh, 1960; Bunning, 1964; and Aschoff, 1965). Furthermore, circadian oscillations have been exploited by organisms for a variety of uses, such as compensation for sun movement in navigation, synchronization of social behavior and measurement of photoperiod; some of these uses are surely unrelated to the original sources of selection for circadian oscillations (Hoffman, 1960; Pittendrigh, 1961, 1966). These findings have led some authors to propose that circadian oscillations are an ancient and integral part of eucaryote physiology (Pittendrigh, 1960, 1961, 1966; Halberg, 1960; and Bunning, 1964), and two authors have suggested that circadian oscillations inhere in the organization and reading of the genetic message (Ehret and Trucco, 1967).

On the other hand, it has long been known that many plants and animals which normally manifest circadian rhythms do not manifest them under certain environmental conditions. These types of "arhythmicity" may be divided into two broad categories corresponding to the sequence of experimental conditions which cause it: (1) Primary arhythmicity: Circadian rhythms are not evident and they do not develop if an organism or population of organisms is raised (from seed or egg) in constant temperature and constant light or dark. (2) Secondary arhythmicity: Once induced by a periodic environment (light and/or temperature signals), circadian rhythms can be inhibited by (a) constant very low temperature, (b) an oxygen-depleted atmosphere, or, (c) constant bright light (Bunning, 1964; Wilkins, 1965). In primary arhythmic organisms, a circadian rhythm can be induced by single or repeated signals (light or temperature). And in secondary arhythmic organisms, the circadian rhythm can be restored by return to non-inhibitory conditions (higher temperature, oxygenated atmosphere, dim light).

As several authors have noted (Pittendrigh and Bruce, 1957; Wasserman, 1959; Sweeney and Hastings, 1960), these two types of arhythmicity could be interpreted as due to *asynchrony* or *arhythmicity* of constituent parts (organelles in cells; cells or organs in individuals; individuals in populations); that is, either the constituent parts are not oscillating (arhythmicity), or the constituent parts are oscillating, but with their phases distributed randomly (asynchrony). This paper discusses some old and new facts bearing on these two possible interpretations and the meaning of these facts for any hypothesis to the effect that circadian rhythmicity is an essential component of physiological organization.

First, concerning primary arhythmicity, an experimental distinction between the two interpretations is made possible by the finding that a single light or temperature signal induces a circadian rhythmicity in primary arhythmic individuals and populations (Pittendrigh, 1954; Wilkins, 1965). The question may be restated in terms of this phenomenon: "Is the *induction* of circadian rhythmicity in primary arhythmic populations due to *initiation* of circadian oscillations inherited at rest, or to *synchronization* of circadian oscillations inherited in motion but out of phase?" As Pittendrigh and Bruce (1957) noted, this question may be answered by comparing the effects of a light or temperature signal (a) when it acts to phase shift (reset) a population of circadian oscillations known to be running and synchronized, and (b) when it acts to induce rhythmicity in a primary arhythmic population.

Pittendrigh (1954) found that either a 4 hour light pulse or a 4 hour temperature pulse (16°/26°/16° C) induces a circadian rhythm in adult emergence in primary arhythmic populations of *Drosophila pseudoobscura* pupae.

Later, Pittendrigh and Bruce (1957) compared the inducing and phase shifting effects of light signals on the *Drosophila* rhythm, and found the results in support of the synchronization hypothesis. However, their experiments showed only the possible correctness of the synchronization hypothesis, because the light signals they used can generate up to 12 hour phase shifts; the possibility of rhythm *initiation* by the light signals was not excluded. In fact, concerning rhythm induction by a 4 hour temperature pulse (16°/26°/16° C), Sweeney and Hastings (1960) pointed out that if this temperature pulse generates only small phase shifts when applied to the free running oscillation, then it *a priori* could not synchronize a population of running but randomly phased, 24 hour oscillations. They inferred that the 4 hour temperature pulse generates small phase shifts on the basis of the finding that a temperature step (26°/16° C) generates only small phase shifts when applied to the free running oscillation (Pittendrigh, Bruce and Kaus, 1958).

In this paper a comparison is made between the phase-shifting and inducing effect of the same temperature signal on the emergence rhythm in *Drosophila pseudoobscura* pupae.

MATERIALS AND METHODS

Two types of automatic collection devices have been developed for routine assay of the *Drosophila* emergence rhythm. The first type of collection device was used in the recently reported phase shift experiments (Zimmerman, Pittendrigh and Pavlidis, 1968); its use involves rearing pupae in plastic boxes, collecting them by flotation, and gluing them to a brass plate. For the rhythm induction experiments reported here, a second type of collection device was designed in which the flies undergo their complete life cycle; the collection of pupae by flotation in the light is thus avoided. Each such device consists of 4 hollow-walled lucite cups mounted on a threaded steel rod, and surrounded by a cannister made of 4-inch diameter lucite tubing and a plastic funnel. Parent flies lay eggs on food inside the cups, and later the larvae crawl out and pupate on rug yarn wrapped around the walls of the cup. The cannister surrounding the cups is suspended from a solenoid whose periodic actuation lifts and drops the system, thus shaking flies

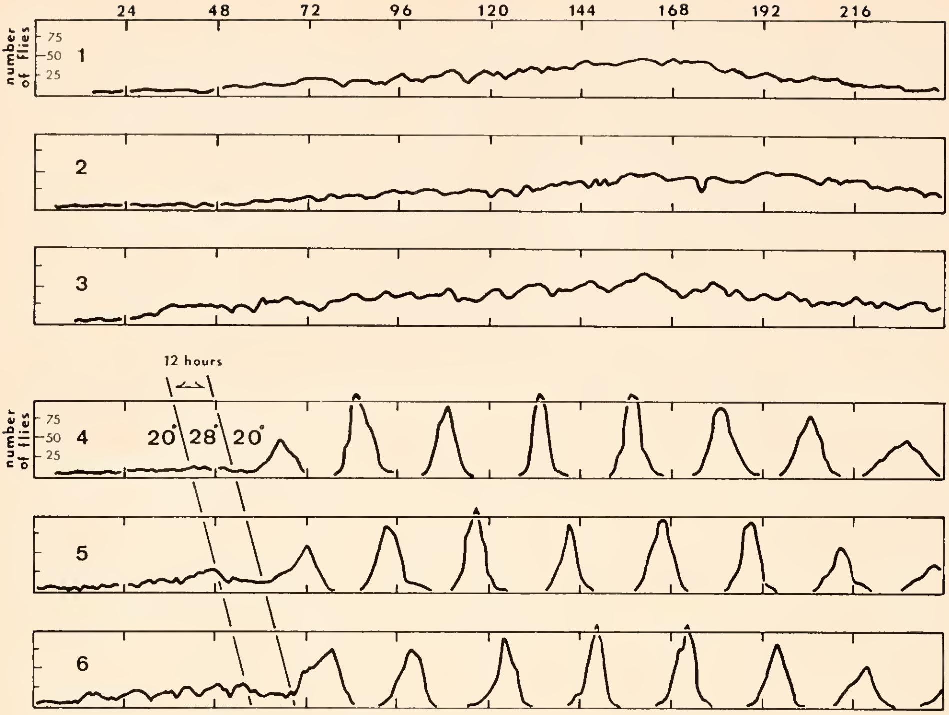

Figure 1. Arhythmicity and the induction of a circadian rhythmicity in populations of *Drosophila pseudoobscura*. Shown are the number of adult flies which emerged per hour over a 10 day period from 6 populations of pupae of mixed developmental ages. The top 3 populations were kept throughout in constant dark and at constant 20° C. The bottom 3 populations were kept in constant dark, but were exposed to a single 12 hour high temperature pulse (12 hours at 28° C); the pulses were started at successive 8 hour intervals (see text).

which have emerged into a vial of detergent solution into which the cannister vents (for details, see Zimmerman, 1966). Temperature control is achieved by pumping water through the hollow-walled lucite cups; each set of cups is coupled via pumps and water valves to two water baths—one at constant 20° C and the other at constant 28° C. Automatic time switches are set to turn on and off the valves and pumps, thus switching the water flow through the cups from one temperature-controlled bath to another. Control of the light regime is provided by 4 watt white fluorescent bulbs connected to time switches. Unlike the collection devices used in the phase shift experiments—which involved collection of pupae by flotation in the light—these new collection devices guarantee constant conditions throughout the organism's life cycle.

RESULTS

Arhythmicity and the induction of rhythmicity

Figure 1 shows the number of flies which emerged per hour for 10 days in 6 populations of *Drosophila pseudoobscura*. The upper 3 populations were kept in constant dark (DD) and constant 20° C throughout (egg to adult); the lower 3 populations were exposed to a single 12 hour high temperature pulse (20°/28°/20° C) after emergence had begun. It is clear that the 3 populations kept throughout in constant conditions are arhythmic: emergence occurs randomly throughout the day. The lower 3 populations were also *initially aperiodic,* but after exposure to the temperature pulse a circadian rhythmicity in adult emergence is induced. Furthermore, the *phase* of the induced emergence rhythm is determined by the time (local) when the pulse was given.

Phase shifting the rhythm by a temperature pulse

In the upper part of Figure 2 is shown the rhythm phase shifting effect of the 12 hour high temperature pulse (20°/28°/20° C): 12 populations of pupae were raised in an LD 12:12 cycle (12 hours light/12 hours dark) at constant 20° C, placed in DD (after the "final dusk"), and exposed to the temperature pulse at successively later times (2 hour intervals). The plotted points are the median hours of the emergence peaks for each day. The dotted vertical lines show the daily median emergence hour of the "free run control"—a population of pupae released into DD and constant 20° C, but *not* subjected to the temperature pulse.

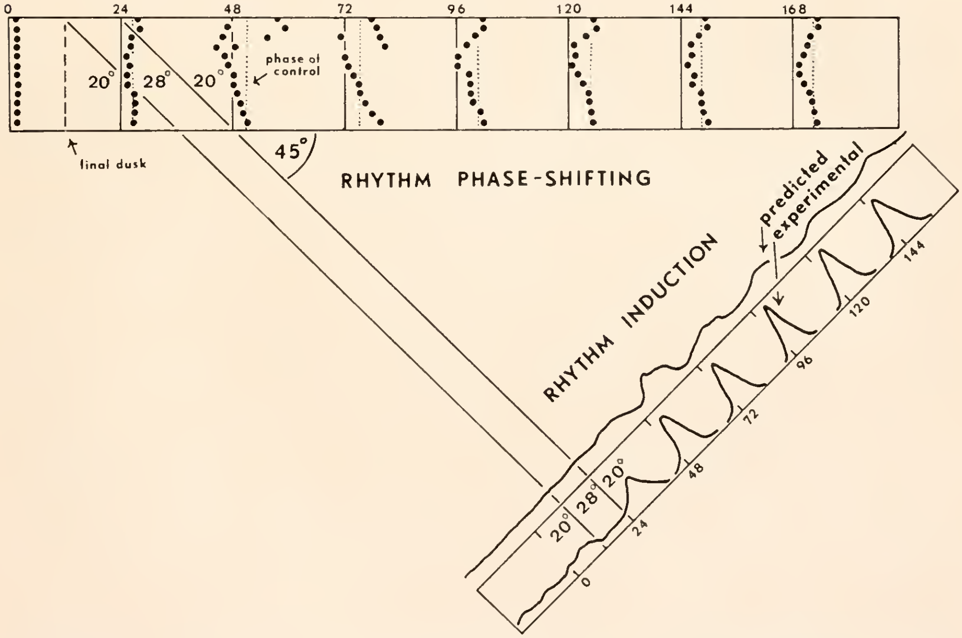

FIGURE 2. Comparison of the rhythm-phase shifting and rhythm-inducing effects of a 12 hour high temperature pulse (20°/28°20° C). In the upper part of the figure are shown the daily median emergence hours of 12 populations of *Drosophila pseudoobscura* exposed to the temperature pulse at successively later times (2 hour intervals). Forty-five degree summation of the number of flies which emerged per hour from these 12 populations yields the "predicted" emergence distribution plotted in the lower part of the figure. The "experimental" distribution was obtained by pooling the lower 3 emergence distributions in Figure 1 (after normalizing these distributions to the onset of the temperature pulse). See text.

Large transient phase shifts are evident on the first and second days after the pulse. However, the final steady state phase shifts—evident on the fifth and sixth days after the pulse—are small; the maximum delay phase shift is 1.3 hours, and the maximum advance phase shift is 3.3 hours. This experiment thus characterizes the magnitude and direction of the phase shift generated by the temperature pulse as a function of the point in the circadian oscillation's cycle exposed to the signal (Zimmerman, Pittendrigh and Pavlidis, 1968; *cf.* Pittendrigh and Minis, 1964).

DISCUSSION

The rhythm phase shifting and rhythm induction effects of the temperature pulse are compared in Figure 2. Forty-five degree projection of the phase shifting data *before the pulse* synthesizes (in the lower part of Figure 2) a model population of pupae which is arhythmic, but which is known to consist of individuals whose oscillations are in motion with phases distributed randomly. Forty-five degree projection of the phase shift data *during and after the pulse* thus simulates the synchronization hypothesis by subjecting the model population of running but asynchronous oscillations to a synchronizing (phase shifting) signal. The prediction resulting from this 45 degree summation of the phase shifting experiment illustrates what is *a priori* clear: a signal which generates steady state phase shifts of only a few hours is incapable of synchronizing a population of running but asynchronous circadian (~24 hour) oscillations.

Below the synthetic distribution—"predicted" from the synchronization hypothesis—is the "experimental" emergence distribution of an arhythmic population of pupae exposed to the temperature pulse; these data are pooled from the lower three experiments shown in Figure 1. The results were previously clear: the temperature pulse does induce a circadian rhythmicity in emergence. The synchronization hypothesis is thus excluded, and we can conclude that the circadian oscillation in individual flies is inherited at rest, and that it is *initiated* (set in motion) by the first light or temperature signal.

Turning now to secondary arhythmicity, the possible interpretations may be formulated in a question analogous to that posed for primary arhythmicity: Is the loss of overt rhythmicity in individuals and populations due to a *damping out* of circadian oscillations in constituent parts, or to a *desynchronization* of circadian oscillations which continue to run in constituent parts? The best experiments bearing on this question are those of Sweeney (1960) on the marine dinoflagellate, *Gonyaulax*. She found that both individual cells and populations of cells show a circadian rhythm if placed in constant dim light (50 footcandles) after a previous LD cycle; however, the rhythm is lost in both individual cells and populations of cells if they are placed in constant bright light (800 footcandles) after a previous LD cycle. Wasserman (1959) showed that the circadian rhythm of leaf movement in the plant *Phaseolus* is accompanied by a parallel circadian rhythm in nuclear volume of epidermal cells; if plants are placed in constant bright light after an LD cycle, both rhythmicities cease. Thus, in these two cases, secondary arhythmicity may be attributed to an arhythmicity of constituent parts.

On the other hand, primary and secondary arhythmicity have been discussed in some cases as due, respectively, to asynchrony and desynchronization of oscillatory constituent parts; but discussion of the evidence is beyond the intended scope of this paper (see Bunning, 1964). My point here is first to emphasize that in several well-studied experimental systems (*Drosophila* emergence and others cited) the absence of overt circadian rhythmicity may be attributed to a true arhythmicity of constituent parts; and second, that this finding, considered in conjunction with the fact that most organisms can reproduce and function normally in aperiodic environments, presents definite difficulties for (a) the general notion that the maintenance and entrainment of circadian oscillations is essential to the normal physiology and development of eucaryotic organisms (Pittendrigh, 1960, 1961; Bunning, 1964), and (b) the more specific hypothesis of Ehret and Trucco (1967) that the mechanism for circadian oscillations inheres in the physical organization—and therefore transcription—of the DNA in eucaryotic cells.

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SUMMARY

1. Although circadian rhythms are systematically and physiologically ubiquitous in eucaryotic organisms, they are not evident under certain experimental conditions. For example, there is no circadian rhythm in adult emergence in populations of *Drosophila pseudoobscura* if the organism is raised in constant dark and temperature.

2. In general, such overt arhythmicity could be interpreted as due to asynchrony or true arhythmicity of constituent parts (organelles in cells; cells and organs in individuals; individuals in populations).

3. In the case of the circadian rhythm of adult emergence in *Drosophila pseudoobscura,* a distinction between these two interpretations of arhythmicity is made possible by comparing the rhythm-phase shifting and rhythm-inducing effect of the same temperature signal. It was concluded that arhythmicity of *Drosophila* populations was due to a true arhythmicity of constituent parts (individual flies).

4. Other experiments are mentioned in which the arhythmicity of populations and individuals is attributable to a true arhythmicity of constituent parts.

5. This finding presents difficulties for hypotheses asserting the importance of circadian rhythmicities in the physiology of eucaryotic organisms.

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